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(54) Title: LYMPHOKINE GENE THERAPY OF CANCER

(57) Abstract

A novel method of tumor immunotherapy is described comprising the genetic modification of cells resulting in the secretion of cytokine gene products to stimulate a patient's immune response to tumor antigens. In one embodiment, autologous fibroblasts genetically modified to secrete at least one cytokine gene product are utilized to immunize the patient in a formulation with tumor antigens at a site other than an active tumor site. In another embodiment, cells genetically modified to express at least one tumor antigen product and to secrete at least one cytokine gene product are utilized in a formulation to immunize the patient at a site other than an active tumor site.

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Lymphokine Gene Therapy of Cancer

BACKGROUND

This application is a continuation-in-part of United States Patent Application Serial No. 07/781,356, filed on October 25, 1991, which is a continuation-in-part of United States Patent Application Serial No. 07/720,872, filed on June 25, 1991, both of which are incorporated herein in their entirety.

Recent advances in our understanding of the lead to the immune system have the 10 biology of identification of important modulators of immune responses, called cytokines (1-3). Immune system modulators produced by lymphocytes are termed lymphokines, a subset of the These agents mediate many of the immune 15 responses involved in anti-tumor immunity. Several of these cytokines have been produced by recombinant DNA methodology and evaluated for their anti-tumor effects. lymphokines and administration of immunomodulators has resulted in objective tumor responses 20 in patients with various types of neoplasms (4-7). However, current modes of cytokine administration are frequently associated with toxicities that limit the therapeutic value of these agents.

For example, interleukin-2 (IL-2) is an important lymphokine in the generation of anti-tumor immunity (4). 25 In response to tumor antigens, a subset of lymphocytes termed helper T-cells secrete small quantities of IL-2. This IL-2 acts locally at the site of tumor antigen stimulation to activate cytotoxic T-cells and natural 30 killer cells which mediate systemic tumor cell destruction. intralesional Intravenous, intralymphatic and clinically administration of IL-2 resulted in has significant responses in some cancer patients (4-6). However, severe toxicities (hypotension and adema) limit 35 the dose and efficacy of intravenous and intralymphatic IL-

2 administration (5-7). The toxicity of systemically administered lymphokines is not surprising as these agents mediate local cellular interactions and they are normally secreted in only very small quantities.

Additionally, other cytokines, such as interleukin-4 (IL-4), alpha interferon (α -INF) and gamma interferon (γ -INF) have been used to stimulate immune responses to tumor cells. Like IL-2, the current modes of administration have adverse side effects.

To circumvent the toxicity of systemic cytokine administration, several investigators have examined intralesional injection of IL-2. This approach eliminates the toxicity associated with systemic IL-2 administration (8,9,10). However, multiple intralesional injections are required to optimize therapeutic efficacy (9,10). Hence, these injections are impractical for many patients, particularly when tumor sites are not accessible for injection without potential morbidity.

An alternative approach, involving cytokine gene 20 transfer into tumor cells, has resulted in significant anti-tumor immune responses in several animal tumor models (11-14). In these studies, the expression of cytokine gene products following cytokine gene transfer into tumor cells has abrogated the tumorigenicity of the cytokine-secreting 25 tumor cells when implanted into syngeneic hosts. The transfer of genes for IL-2 (11,12) γ -INF (13) or interleukin-4 (IL-4) (14)significantly reduced or eliminated the growth of several different histological types of murine tumors. In the studies employing IL-2 gene 30 transfer, the treated animals also developed systemic antitumor immunity and were protected against subsequent tumor challenges with the unmodified parental tumor (11,12). Similar inhibition of tumor growth and protective immunity was also demonstrated when immunizations were performed

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with a mixture of unmodified parental tumor c lls and genetically modified tumor cells engineered to express the IL-2 gene. No toxicity associates with localized lymphokine transgene expression was reported in these animal tumor studies (11-14).

while the above gene-transfer procedure has been shown to provide anti-tumor immunity, it still retains practical difficulties. This approach is limited by the inability to transfer functional cytokine genes into many patients' tumor cells, as most patients' tumors cannot be established to grown in vitro and methods for human in vivo gene transfer are not available.

SUMMARY OF THE INVENTION

The present invention demonstrates a novel, more 15 practical method of cytokine cancer immunotherapy. In one approach, selected cells from a patient, fibroblasts, obtained, for example, from a routine skin biopsy, are genetically modified to express one or more cytokines. Alternatively, patient cells which may normally 20 serve as antigen presenting cells in the immune system such as macrophages, monocytes, and lymphocytes may also be genetically modified to express one or more cytokines. These modified cells are hereafter called cytokineexpressing cells, ore CE cells. The CE cells are then 25 mixed with the patient's tumor antigens, for example in the form of irradiated tumor cells, or alternatively in the form of purified natural or recombinant tumor antigen, and employed in immunizations, for example subcutaneously, to induce systemic anti-tumor immunity.

The cytokines are locally expressed at levels sufficient to induce or augment systemic anti-tumor immune responses via local immunization at sites other than active tumor sites. Systemic toxicity related to cytokine

administration should not occur because the levels of cytokine secreted by the CE cells should not significantly affect systemic cytokine concentrations.

As the amount of cytokine secreted by the CE 5 cells is sufficient to induce anti-tumor immunity but is too low to produce substantial systemic toxicity, this local cytokine provides benefit of the approach In addition, this novel method obviates administration. the need for intralesional injections, which may produce 10 morbidity. Furthermore, the continuous local expression of cytokine(s) at the sites of immunization may also augment immune responses compared to intermittent anti-tumor This method also provides the cytokine injections. advantage of local immunization with the CE cells, as 15 opposed to cumbersome intravenous infusions. This method also eliminates the need for establishing tumor cell lines in vitro as well as transfer of genes into these tumor cells.

This invention also provides an alternative means 20 of localized expression of cytokines to induce and/or increase immune responses to a patient's tumor through genetic modification of cellular expression of both In this embodiment, cytokine(s) and tumor antigen(s). selected cells from a patient are isolated and transduced 25 with cytokine gene(s) as well as gene(s) coding for tumor The transduced cells are called "carrier antigen(s). cells." Carrier cells can include fibroblasts and cells which may normally serve as antigen presenting cells in the monocytes, and such as macrophages, system 30 lymphocytes. Transduced carrier cells actively expressing both the cytokine(s) and the tumor antigen(s) are selected and utilized in local immunizations at a site other than active tumor sites to induce anti-tumor immune responses. As with the CE cells, these carrier cells should not produce substantial systemic toxicities, as the levels of cytokine(s) secreted by the carrier cells should not significantly affect systemic cytokine concentrations. This alternate embodiment is advantageous because it obviates the need to obtain samples of the tumor, which is sometimes difficult. However, carrier cells can be utilized in local immunizations in conjunction with tumor cells, tumor cell homogenates, purified tumor antigens, or recombinant tumor antigens to enhance anti-tumor immunity.

additionally, this second embodiment retains the same advantages as the first embodiment in that the level of cytokine released by the carrier cells is sufficient to induce anti-tumor immunity but is too low to produce substantial systemic toxicity. In addition, as with the first embodiment, this method obviates the need for intralesional injections, and allows for continuous expression of cytokine(s). This method also eliminates the need for establishing continuous cultures in vitro of tumor cells as well as transfer of genes into these tumor cells, and provides the advantage of local immunization with the carrier cells, as opposed to cumbersome lengthy intravenous infusions.

These approaches may also find application in inducing or augmenting immune responses to other antigens of clinical significance in other areas of medical practice.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematic diagrams of retroviral vectors DC/TKIL2, LXSN-IL2, and LNCX-IL2.

Figure 2 shows a mean IL-2 concentration of triplicate supernatant samples measured by BLISA. Supernatants were harvested from overnight cultures of approximately 1.5 x 106 semi-confluent fibroblasts.

Figure 3 shows biological activity of the IL-2 secret d by the transduced fibroblasts was demonstrated by measuring mean ³H-TdR incorporation of an IL-2 dependent T-cell line incubated with triplicate samples of supernatants. Supernatants were harvested from overnight cultures of approximately 1.5 x 10⁶ semi-confluent fibroblasts.

Figure 4 shows comparisons between animals injected with 10⁵ CT26 tumor cells alone (\square); 10⁵ CT26 tumor cells and 2 x 10⁶ unmodified BALB/C fibroblasts (\blacksquare); 10⁵ CT26 tumor cells and 2 x 10⁶ IL-2 transduced BALB/C fibroblasts (\blacksquare); and 10⁵ CT26 tumor cells and 1 x 10⁶ transduced BALB/C fibroblasts (\bigcirc). Tumor measurements are the mean products of the cross-sectional diameter of the tumors from four animals in each treatment group. The (*) indicates statistically significant difference (P < 0.05) in tumor growth curves.

Figure 5 shows PCR analysis of neomycin phosphotransferase DNA sequences. Lane 1 - positive control pLXSN-RI-IL2. Lanes 2 through 4 tests genomic DNA; Lanes 5 and 6 ovary genomic DNA; Lane 7 negative control, no DNA. Identical results were obtained with liver, spleen and lung genomic DNA (data not shown).

Figure 6 shows the effect of IL-2 modified 25 fibroblasts on tumor establishment and development using 2 x 10' fibroblasts mixed with 5 x 10' CT26 tumor cells concentrating on the rate of tumor growth.

Figure 7 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2 30 x 10⁶ fibroblasts mixed with 5 x 10⁴ CT26 tumor cells concentrating on the time of tumor onset for the individual animal in each treatment group.

Figure 8 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2 x 10^6 fibroblasts mixed with 1 x 10^5 CT26 tumor cells concentrating on the rate of tumor growth.

- Figure 9 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2 x 10⁶ fibroblasts mixed with 1 x 10⁵ CT26 tumor cells concentrating on the time of tumor onset for the individual animal in each treatment group.
- 10 Figure 10 shows the effect of IL-2 modified cells on tumor establishment and development using 2 x 10⁶ DCTK-IL2-modified CT26 tumor cells mixed with 1 x 10⁵ unmodified CT26 compared to 2 x 10⁶ DCTK-IL2-modified fibroblasts mixed with 1 x 10⁵ CT26 concentrating on the rate of tumor growth.
- 15 Figure 11 shows the effect of IL-2 modified cells on tumor establishment and development using 2 x 10° DCTK-IL2-modified CT26 tumor cells mixed with 1 x 10° unmodified CT26 compared to 2 x 10° DCTK-IL2-modified fibroblasts mixed with 1 x 10° CT26 concentrating on the time of tumor onset 20 for the individual animal in each treatment group.

Figure 12 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the rate of tumor growth. Mice were immunized with 2 x 10⁶ fibroblasts mixed with 2.5 x 10⁵ irradiated CT26 tumor cells 7 days prior to challenge with 5 x 10⁴ fresh tumor cells.

Figure 13 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the time of tumor onset for the individual animal in each treatment group. Mice were immunized with 2 x 10° fibroblasts mixed with 2.5 x 10° irradiated CT26 tumor cells 7 days prior to challenge with 5 x 10° fresh tumor c IIs.

Figure 14 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the rate of tumor growth. Mice were immunized with 2 x 10⁶ fibroblasts mixed with 2.5 x 10⁵ irradiated CT26 tumor cells 14 days prior to challenge with 5 x 10⁴ fresh tumor cells.

Figure 15 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the time of tumor onset for the individual animal in each treatment group. Mice were immunized with 2 \times 10 $^{\circ}$ fibroblasts mixed with 2.5 \times 10 $^{\circ}$ irradiated CT26 tumor cells 14 days prior to challenge with 5 \times 10 $^{\circ}$ fresh tumor cells.

DETAILED DESCRIPTION

novel method of tumor immunotherapy is 15 described comprising the genetic modification of cells resulting in the secretion of cytokine gene products to stimulate a patient's immune response to tumor antigens. "Gene" is defined herein to be a nucleotide sequence encoding the desired protein. In one embodiment, 20 autologous fibroblasts genetically modified to secrete at least one cytokine gene product are utilized to immunize the patient in a formulation with tumor antigens at a site other than an active tumor site. In another embodiment, cells genetically modified to express at least one tumor antigen gene product and to secrete at least one cytokine gene product are utilized in formulation to immunize the patient at a site other than an active tumor site. Cytokines are preferably expressed in cells which efficiently secrete these proteins into the surrounding 30 milieu. fibroblasts are an example of such cells. Fibroblasts or other cells can be genetically modified to express and secrete one or more cytokines, as described later in this specification.

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Tumor antigens can be provided by several methods, including, but not limited to the following: 1) CE cells can be transduced with gene(s) coding for tumor These "carrier cells" are then utilized in antigens. 5 patient immunizations. 2) Cloned gene sequences coding for appropriate tumor antigens can be transferred into cells such as fibroblasts or antigen-presenting cells. cells are then mixed with CE or carrier cells to immunize the patient. 3) Tumor antigens can be cloned in bacteria 10 or other types of cells by recombinant procudures. These antigens are then purified and employed an immunization with CE and/or carrier cells. 4) Tumor antigens can be purified from tumor cells and used, along with CE or carrier cells, to immunize the patient. 5) Tumor cells may 15 be irradiated or mechanically disrupted and mixed with CE and/or carrier cells for patient immunizations.

This invention encompasses the following steps: (A) isolation of appropriate cells for generation of CE cells or carrier cells; (B) isolation of cytokine genes or 20 isolation of cytokine genes and tumor antigen genes, as well as appropriate marker and/or suicide genes; (C) transfer of the genes from (B) to produce the CE cells or carrier cells; (D) preparation of immunological samples of the patient's tumor antigens or other suitable tumor antigens for immunization with CE or carrier cells; (E) inactivation of the malignant potential of tumor cells if are used as a source of tumor antigens for immunization; and (F) preparation of samples for immunization. Following are several embodiments 30 contemplated by the inventors. However, it is understood that any means known by those in the art to accomplish these steps will be usable in this invention.

(A) <u>Isolation of Cells to Generate CE and</u> <u>Carrier Cells</u>

Cells to be utilized as CE cells and carrier cells can be selected from a variety of locations in the patient's body. For example, skin punch biopsies provide a readily available source of fibroblasts for use in generating CE cells, with a minimal amount of intrusion to the patient. alternatively, these fibroblasts can be obtained from the tumor sample itself. Cells of hematopoietic origin may be obtained by venipuncture, bone marrow aspiration, lymph node biopsies, or from tumor samples. Other appropriate cells for the generation of CE or carrier cells can be isolated by means known in the art. Non-autologous cells similarly selected and processed can also be used.

(B) <u>Isolation of Genes</u>

Numerous cytokine genes have been cloned and are available for use in this protocol. The genes for IL-2, γ-INF and other cytokines are readily available (1-5, 11-14). Cloned genes of the appropriate tumor antigens are isolated according to means known in the art.

Selectable marker genes such as neomycin resistance (Neok) are readily available. Incorporation of a selectable marker gene(s) allows for the selection of cells that have successfully received and express the desired genes. Other selectable markers known to those in the art of gene transfer may also be utilized to generate CE cells or carrier cells expressing the desired transgenes.

"Suicide" genes can be incorporated into the CE cells or carrier cells to allow for selective inducible killing after stimulation of the immune r spons . A g ne

such as the herpes simplex virus thymidine kinase gene (TK) can be used to create an inducible destruction of the CE cells or carrier cells. When the CE cells or carrier cells are no longer useful, a drug such as acyclovir or 5 gancyclovir can be administered. Either of these drugs will selectively kill cells expressing TK, thus eliminating the implanted transduced cells. Additionally, a suicide gene may be a gene coding for a non-secreted cytotoxic polypeptide attached to an inducible promoter. When 10 destruction of the CE or carrier cells is desired, the appropriate inducer of the promoter is administered so that the suicide gene is induced to produce cytotoxic polypeptide which subsequently kills the CE or carrier cell. However, destruction of the CE or carrier cells may 15 not be required.

Genes coding for tumor antigen(s) of interest can be cloned by recombinant methods. The coding sequence of an antigen expressed by multiple tumors may be utilized for many individual patients.

20 (C) Transfer of Genes

Numerous methods are available for transferring genes into cultured cells (15). For example, the appropriate genes can be inserted into vectors such as plasmids or retroviruses and transferred into the cells.

25 Electroporation, lipofection and a variety of other methods are known in the field and can be implemented.

One method for gene transfer is a method similar to that employed in previous human gene transfer studies, where tumor infiltrating lymphocytes (TILs) were modified by retroviral gene transduction and administered to cancer patients (16). In this Phase I safety study of retroviral mediated gene transfer, TILs were genetically modified to express the Neomycin resistance (Neo^R) gene. Following

intravenous infusion, polymerase chain reaction analyses consistently found genetically modified cells in the circulation for as long as two months after administration. No infectious retroviruses were identified in these patients and no side effects due to gene transfer were noted in any patients (16). These retroviral vectors have been altered to prevent viral replication by the deletion of viral gag, pol and env genes.

When retroviruses are used for gene transfer, 10 replication competent retroviruses may theoretically develop by recombination between the retroviral vector and viral gene sequences in the packaging cell line utilized to produce the retroviral vector. We will use packaging cell lines in which the production of replication competent 15 virus by recombination has been reduced or eliminated. Hence, all retroviral vector supernatants used to infect patient cells will be screened for replication competent virus by standard assays such as PCR and reverse transcriptase assays (16). Furthermore, exposure to 20 replication competent virus may not be harmful. In studies of subhuman primates injected with a large inoculum of replication competent murine retrovirus, the retrovirus was cleared by the primate immune system (17). No clinical illnesses or sequelae resulting from replication competent 25 virus have been observed three years after exposure. summary, it is not expected that patients will be exposed to replication competent murine retrovirus and it appears that such exposure may not be deleterious (17).

(D) <u>Preparation of Immunological Samples of the</u>
<u>Patient's Tumor Antigens or Purified</u>
<u>Recombinant Tumor Antigens</u>

Tumor cells bearing tumor associated antigens are isolated from the patient. These cells can derive either from solid tumors or from leukemic tumors. Fr solid

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tumors, single-cell suspensions can be made by mechanical separation and washing of biopsy tissue (18).

Hematopoietic tumors may be isolated from peripheral blood or bone marrow by standard methods (19).

A second variant is the use of homogenates of tumor cells. Such homogenates would contain tumor antigens available for recognition by the patient's immune system upon stimulation by this invention. Either unfractionated cell homogenates, made, for example, by mechanical disruption or by freezing and thawing the cells, or fractions of homogenates preferably with concentrated levels of tumor antigens, can be used.

Likewise, purified tumor antigens, obtained for example by immunoprecipitation or recombinant DNA methods, could be used. Purified antigens would then be utilized for immunizations together with the CE cells and/or carrier cells described above to induce or enhance the patient's immune response to these antigens.

In the embodiments employing carrier cells, tumor antigens are available through their expression by the carrier cells. These carrier cells can be injected alone or in conjunction with other tumor antigen preparations or CE cells. Likewise, when CE cells are used, purified recombinant tumor antigen, produced by methods known in the art, can be used.

If autologous tumor cells are not readily available, heterologous tumor cells, their homogenates, their purified antigens, or carrier cells expressing such antigens could be used.

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(E) <u>Inactivation of Tumor Cells</u>

When viable tumor cells are utilized in immunizations as a source of tumor antigens, the tumor cells can be inactivated so that they do not grow in the patient. Inactivation can be accomplished by several methods. the cells can be irradiated prior to immunization (18). This irradiation will be at a level which will prevent their replication. Such viable calls can then present their tumor antigens to the patient's immune system, but cannot multiply to create new tumors.

Alternatively, tumor cells that can be cultured may be transduced with a suicide gene. As described above, a gene such as the herpes simplex thymidine kinase (TK) gene can be transferred into tumor cells to induce their 15 destruction by administration of acyclovir or gancyclovir. After immunization, the TK expressing tumor cells can present their tumor antigens, and are capable proliferation. After a period of time during which the patients's immune response is stimulated, the cells can be 20 selectively killed. This approach might allow longer viability of the tumor cells utilized for immunizations, which may be advantageous in the induction or augmentation of anti-tumor immunity.

(F) Preparation of Samples for Immunization

CE cells and/or carrier cells and tumor cells, and/or homogenates of tumor cells and/or purified tumor antigen(s), are combined for patient immunization. Approximately 10' tumor cells will be required. If homogenates of tumor cells or purified or non-purified fractions of tumor antigens are used, the tumor dose can be adjusted based on the normal number of tumor antigens usually present on 10' intact tumor cells. The tumor preparation should be mixed with numbers of CE or carrier

cells sufficient to secrete cytokine levels that induce anti-tumor immunity (11-12) without producing substantial systemic toxicity which would interfere with therapy.

The cytokines should be produced by the CE cells or the carrier cells at levels sufficient to induce or augment immune response but low enough to avoid substantial systemic toxicity. This prevents side effects created by previous methods' administration of greater than physiological levels of the cytokines.

These mixtures, as well as carrier cells that are utilized alone, will be formulated for injection in any manner known in the art acceptable for immunization. Because it is important that at least the CE cells and carrier cells remain viable, the formulations must be compatible with cell survival. Formulations can be injected subcutaneously, intramuscularly, or in any manner acceptable for immunization.

Contaminants in the preparation which may focus the immune response on undesired antigens should be removed prior to the immunizations.

The following examples are provided for illustration of several embodiments of the invention and should not be interpreted as limiting the scope of the invention.

EXAMPLE I

IMMUNIZATION WITH FIBROBLASTS EXPRESSING IL-2 MIXED WITH IRRADIATED TUMOR CELLS

1) Isolation of Autologous Fibroblasts for Use in Generating IL-2 Secreting CE Cells

Skin punch biopsies will be obtained from each patient under sterile conditions. The biopsy tissue will be minced and placed in RPMI 1640 media containing 10% fetal calf serum (or similar media) to establish growth of the skin fibroblasts in culture. The cultured fibroblasts will be utilized to generate IL-2 secreting CE cells by retroviral mediated IL-2 gene transfer.

2) Retroviral Vector Preparation and Generation of IL-2 Secreting CE Cells

15 The cultured skin fibroblasts will infected with a retroviral vector containing the IL-2 and Neomycin resistance (NeoR) genes. An N2 vector containing the Neo* gene will be used, and has been previously utilized by a number of investigators for in vitro and in vivo work, 20 including investigations with human subjects (16). The IL-2 vector will be generated from an N2-derived vector, LLRNL, developed and described by Friedmann and his colleagues (20). It will be made by replacement of the luciferase gene of LLRNL with a full-length cDNA encoding 25 human IL-2. Retroviral vector free of contaminating replication-competent virus is produced by transfection of vector plasmid constructions into the helper-free packaging cell line PA317. Before infection of patients' cells, the vector will have been shown to be free of helper virus. In 30 the event that helper virus is detected, the vector will be produced in the GP + envAM12 packaging cell line in which

the viral gag and pol genes are separated from the env, further reducing the likelihood of helper virus production.

3) Transduction Protocol

primary fibroblasts will cultured 5 incubated with supernatant from the packaging cell line as Supernatant from these cells will be described (20). tested for adventitious agents and replication competent virus as described (16) and outlined in Table 1. fibroblasts are washed and then grown in culture media 10 containing G418, (a neomycin analogue) to select for transduced cells expressing the NeoR gene. The G418resistant cells will be tested for expression of the IL-2 gene by measuring the concentration of IL-2 in the culture supernatant by an enzyme linked immunosorbent assay (ELISA) 15 (12). G418-resilient cells expressing IL-2 will be stored subsequent -70°C until required for at immunizations.

Table 1

Adventitious Agents and Safety Testing Sterility 20 1. 2. Mycoplasma 3. General Safety 4. Viral Testing LCM Virus 25 Thymic agent S+/L- eco S+/L-xeno S+/L- ampho 3T3 amplification 30 MRC-5/Vero

4) Preparation of Irradiated Tumor Cells

obtained form clinically indicated Tumors surgical resections or from superficial lymph node or skin metastases will be minced into 2-3 mm pieces and treated 5 with collagenase and DNAse to facilitate separation of the tumor into a single cell suspension. The collected cells will be centrifuged and washed in RPMI 1640 media and then cryopreserved in a solution containing 10% dimethyl sulphoxide and 50% fetal calf serum in RPMI 1640 media. 10 The cells will be stored in liquid nitrogen until the time Prior to their use in subcutaneous of administration. immunizations, the cells will be thawed, washed in media free of immunogenic contaminants, and irradiated with 4,000 rads per minute for a total of 20,000 rads in a cesium 15 irradiator.

5) Patient Selection

Patients will have a histologically confirmed diagnosis of cancer. Patients with tumors that must be resected for therapeutic purposes or with tumors readily accessible for biopsy are most appropriate for this embodiment of the invention.

6) Pretreatment Evaluation

The following pretreatment evaluations will be performed:

25 1) History and physical examination including a description and quantification of disease activity.

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- 2) Performance Status Assessment
 - 0 = Normal, no symptoms
 - l = Restricted, but ambulatory
 - 2 = Up greater than 50% of waking hours, capable of self-care
 - 3 = Greater than 50% of waking hours
 confined to bed or chair, limited
 self-care
 - 4 = Bedridden
- 10 3) Pretreatment Laboratory:

CBC with differential, platelet count, PT, PTT, glucose, BUN, creatinine, electrolytes, SGOT, SGPT, LDH, alkaline phosphatase, bilirubin, uric acid, calcium, total protein albumin.

15 4) Other Analyses: Urinalysis

CH₅₀, C₁ and C₄ serum complement levels

Immunophenotyping of peripheral blood B cell and
T cell subsets

Assays for detectable replication-competent virus in peripheral blood cells

PCR assays of peripheral blood leukocytes for Neo^R, IL-2 and viral env

- 5) Other Pretreatment Evaluation:
- Chest X-ray and other diagnostic studies including computerized tomography (CT), magnetic resonance imaging (MRI) or radionuclide scans may be performed to document and quantify the extent of disease activity.
- Follow-up evaluations of these assessments at 30 regular intervals during the course of therapy (approximately every 1 to 3 months) will be useful in determining response to therapy and potential toxicity,

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permitting adjustments in the number of immunizations administered.

7) Restrictions on Concurrent Therapy

For optimal effects of this treatment, patients should receive no concurrent therapy which is known to suppress the immune system.

8) Final Formulation

Each patient will receive subcutaneous immunizations with a mixture if irradiated tumor cells and autologous fibroblast CE cells genetically modified to secrete IL-2. Approximately 10' tumor cells will be mixed with 10' fibroblasts known to secrete at least 20 units/ml of IL-2 in tissue culture when semi-confluent (12). The irradiated tumor cells and genetically modified fibroblasts will be placed in a final volume of 0.2 ml normal saline for immunization.

9) Dose Adjustments

At least two subcutaneous immunizations will be administered, two weeks apart, with irradiated tumor cells and autologous fibroblasts genetically modified to secrete IL-2. If no toxicity is observed, subsequent booster immunizations may be administered periodically (at least one week apart) to optimize the anti-tumor immune response.

J) Treatment of Potential Toxicity

Toxic side effects are not expected to result from these immunizations. However, potential side effects of these immunizations are treatable in the following manner:

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If massive tumor cell lysis results, any resulting uric acid nephropathy, adult respiratory distress syndrome, disseminated intravascular coagulation or hyperkalemia will be treated using standard methods.

Local toxicity at the sites of immunization will be treated with either topical steroids and/or surgical excision of the injection site as deemed appropriate.

Hypersensitivity reactions such as chills, fever and/or rash will be treated symptomatically with antipyretics and antihistamines. Patients should not be treated prophylactically. Should arthralgias, lymphadenopathy or renal dysfunction occur, treatment with corticosteroids and/or antihistamines will be instituted. Anaphylaxis will be treated by standard means such as administration of epinephrine, fluids, and steroids.

EXAMPLE II

A. Retroviral IL-2 Gene Transfer and Expression in Fibroblasts

Retroviral vectors were employed to transfer and 20 express IL-2 and neomycin phosphotransferase genes in murine and primary human fibroblasts. The retroviral DC/TKIL2 produced by Gilboa and (Gansbacher, et al., J. Exp. Med. 172:1217-1223, 1990, which is incorporated herein by reference) was utilized to 25 transduce murine fibroblasts for application in an animal tumor model (see Section B below). Human fibroblasts were transduced with the retroviral vector LXSN-RI-IL2. Schematic diagrams of the structure of these retroviral vectors are provided in Figure 1. A more complete 30 description of the LXSN-RI-IL2 vector, including its nucleotide sequence, is provided in Example III and in Tables 2, 3 and 4.

Following infection with the described vectors and selection for 2-3 weeks in growth media containing the neomycin analogue G418, Balb/c and human embryonic fibroblast culture supernatants were harvested and tested for IL-2 by an enzyme-linked immunosorbent assay (ELISA). Figure 2 depicts the levels of IL-2 secreted by the transduced fibroblasts.

These results can be confirmed using negative control fibroblasts infected with an N2-derived retroviral vector expressing an irrelevant gene such as luciferase or ß-galactosidase and studies with adult human fibroblasts.

Biological activity of the IL-2 expressed by the transduced human fibroblasts was confirmed by a cell proliferation bioassay employing an IL-2 dependent T cell line. In this assay, supernatant from the transduced fibroblasts and control unmodified fibroblasts were incubated with the IL-2 dependent T cell line CTIL-2. Incorporation of 'H-thymidine was measured as an indicator of cell proliferation and IL-2 activity (Figure 3).

20 B. <u>Efficacy of Transduced Fibroblasts in an Animal Tumor Model</u>

The efficacy of fibroblasts genetically modified to secrete IL-2 was tested in an animal model of colorectal carcinoma. In these studies, the Balb/c CT26 tumor cell line was injected subcutaneously with Balb/c fibroblasts transduced to express IL-2. Control groups included animals injected with 1) a mixture of CT26 tumor cells and unmodified fibroblasts; 2) CT26 tumor cells without fibroblasts and 3) transduced fibroblasts alone. No tumors were detected in 3/8 animals treated with transduced fibroblasts and CT26 cells. In contrast, all untreated control animals (8/8) injected with CT26 tumor cells dev loped palpable tumors. No tumors were detect d in the

animals inoculated with transduced fibroblasts without CT26 The mean CT26 tumor size in Balb/c mice tumor cells. injected with the IL-2 secreting fibroblasts was considerably smaller compared to the control groups (Figure 5 4). A multivariate non-parametric statistical procedure (Koziol, et al., Biometries 37:383-390, 1981 and Koziol, et al., Computer Prog. Biomed. 19:69-74, 1984, which is incorporated herein by reference) was utilized to evaluate differences in tumor growth among the treatment groups. 10 The tumor growth curves for the four treatment groups presented in Figure 4 were significantly different (p=0.048). Subsequent comparisons between treatment groups revealed a significant difference (p < 0.05) in tumor growth between animals injected with CT26 tumor cells alone 15 and animals treated with 2 x 106 transduced fibroblasts and CT26 tumor cells (Figure 4).

EXAMPLE III

A. Project Overview

Lymphokine gene therapy of cancer will be 20 evaluated in cancer patients who have failed conventional An N2-derived vector containing the neomycin phosphotransferase gene will be used. This vector has been employed by a number of investigators for in vitro and in vivo studies including recently approved investigations 25 with human subjects (Rosenberg et al., N. Eng. J. Med., 323:570-578, 1990). The lymphokine vectors used in this investigation will be generated from the N2-derived vector, LXSN, developed and described by Miller et al., Mol. Cell Biol. 6:2895, 1986 and Miller et al., BioTechniques 7:980, 1989, which are incorporated herein by reference. The vector LXSN-RI-IL2 contains human IL-2 cDNA under the control of the retroviral 5' LTR promoter and the neomycin phosphotransferase gene under the control of the SV40 promoter (see Figure 1). The normal human IL-2 leader sequence has been replaced with a chimeric sequence containing rat insulin and human IL-2 leader sequences (see Tables 2, 3 and 4). This chimeric leader sequence enhances IL-2 gene expression.

To construct the LXSN-RI-IL2 vector, the bacterial plasmid pBC12/CMV/IL2 (Cullen, B.R., DNA 7:645-650, 1988, which is incorporated herein by reference) containing the full-length IL-2 cDNA and chimeric leader sequence was digested with **HindIII** and the ends were blunted using Klenow polymerase. IL-2 cDNA was subsequently released from the plasmid by digestion with The IL-2 fragment was purified by electrophoresis in a 1% agarose gel and the appropriate band was extracted utilizing a glass powder method. Briefly, the gel slice was dissolved in 4M NaI at 55°. After cooling to room temperature, 4 μ l of oxidized silica solution (BIO-101, La Jolla, CA) was added to adsorb the DNA. The silica was ythen washed with a cold solution of 50% ethanol containing 0.1 M NaCl in TE buffer. The DNA was eluted from the 20 silica by heating at 55° in distilled H2O. The purified IL-2 cDNA was then directionally ligated into the HpaI-BamHI cloning sites of the pLXSN vector. A more complete description of the pLXSN-RI-IL2 vector and its partial nucleotide sequence are provided in Tables 2, 3, 4, 5 and 25 6.

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Table 2

Description of the LXSN-RI-IL2 from position 1 to 6365

Bases	Description
1-589	Moloney murine sarcoma virus 5° LTR
659-1458	The sequence of the extended packaging signal
1469-2151	IL-2 cDNA with chimeric leader sequence
1469-1718	IL-2 chimeric leader sequence
1647-1718	coding region of the signal peptide
1719-2151	Mature IL-2 coding sequence
2158-2159	Mo mu sarcoma virus end/SV 40 start
2159-2503	Simian virus 40 early promoter
2521-2522	Simian virus DNA end/TnS DNA start
2557-3351	Neomycin phosphotransferase
3370-3371	TnS DNA end/Moloney murine leukemia virus start
3411-4004	Moloney murine leukemia virus 3' LTR
4073-4074	Moloney murine leukemia DNA end/pBR322 DNA start
4074-6365	Plasmid backbone

Table 3

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Table 4

Enzymes which do not cut LXSNRII.L2:

Acc3 SnaBl	Bgl2	Clal	Hpal	Nrul
Apal Spll	Bsm1	Dra3	Mlul	PflMl
Asu2 Sst2	BspM2	Eco47III	Mrol	Sac2
Ban3	BstBl	Espl	Not1	Sall

SUBSTITUTE SHEET

---1---1-2-+--23--31---+--3-----+1---

--1--1

Apall Apyl Agul Asel

Alwni

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Aoc1 Aoc2 Aos1 Asp700

Table 5

Numbered from position 1

From 1 to 6365.

----1000+----2000+----3000+---

LXSNRII.L2

211-1-12--+----2--1--2----2--1---1---1-112--1-+12--1--12--+1-----111--+--------1 -----1 Mo-MuSV 5' long ter neomycin phosphotra 3' long ter 1 to 683 of RIIL2 signal Aat2 Aha2 Aha3 Aat1 Af12 Af13 Acc2 Alul Acyl Afil Aha1 Acc 1 [Split] Mo-MuLV

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Table 5

from 1 to 6365. Numbered from position 1.

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-----1000+-------6000+------6000+------1000+-------6000+-------6000+-------1----177 -12+1--1-1-1-1-------Ì ---12-1---1 --2-7-Table 5 (Cont'd) --111-11--1-1-1---5----------Munbered from position 1. ---11-1-------1111-1-----Mady 8' long ter --------11---necesseia phosphotra LICHALILY -Nully 3' long ter l to 600 of alles Beplaser fra 1 to 1565. Asp718 olgnel Aepa1 1 APLI Ave 1 Aves Aves Aves 1 3 34 74 1011 2011 (spite)

Table 5 (Cont'd)

from 1 to 6365. Numbered from position 1.

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Table 5 (Cont'd)

from 1 to 6365. Numbered from position 1.

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Table 5 (Cont'd)

A from 1 to 6365. Humbered from position 1. -Muffy 5' long ter ----received phesphotra
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from 1 to 6365. Numbered from position 1.

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Table 6 (Cont'd)

>Set1 >BeeH2 >RgiAl 370 380 • • TTATTTGAAC TAACCAATCA GITCCCTTCT COCTTCTGTT CCCCCCCCTTC CCCTCTCCCA CCTCAATAAA ANTANACTIC ATTECTTACT CAACOCAACA COCCACACAC COCACACCCT CCACTTATT - 2006 >Aep718 >2041 >Ban2 >300E2 >Mgal >Tth1111 470 AGAGEOGRACA ACCOCTCACT COCCOCCCA STCTTCCCAT AGACTGCCTC GCCCCCGTAC CCCTATTCCC A 4.A A . TCTOCCCTCT TCCCCACTCA COCCCCCCT CACAACCCTA TCTCACCCAC CCCCCCATC CCCATAACCC >Styl 510 . STATITOCCA GAACCACAA CETACCCTTA CCACCACACC GACAACCAAC CCTCCCACAC CACACTCACT >Ban2 TTEACTACOC ACCACCCCC TCTTTCATTT CCCCCCTCCT CCCCCATTTG CACACCCCTG CCCACCCACC AACTGATGGG TGCTGCCCCC AGAAAGTAAA CCCCCCAGCA GGCCCTAAAC CTCTGCGGAC GGGTCCCTGG 240 HEET. HEART

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- 12201

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- 2.4.2. - 2.4.1.1

>BeuleI >Aool >Baul >BcollI

50 WO 93/07906 PCT/US92/08999 Table 6 (Cont'd) >Cvn1 >Xet2 >Ple1 >Tth1111 • 5. N.E. 860 1880 490 100 • • • • • • CCCCACCTCA GGAAGGGAGT GGATGTGGAA TCCGACCCCC TCAGGATATG TGGTTCTGGT AGGAGACCAG COGCTOGACT CCTTCCCTCA GCTACACCTT AGGCTGCCGC AGTCCTATAC ACCAAGACCA TCCTCTGCTC >Hgal 920 730 940 950 960 • • AACCTAAAAC AGTTCCCCCC TCCCTCTCAA TTTTTCCTTT CCCTTTCCAA CCCAAGCCCCC CCCTCTTCTC TTGGATTTTG TCAAGGGGGG AGGCAGACTT ALLACGAA GCCAAACCTT GGCTTCGGCG CGCAGAACAG >Pet1 1010 1020 1030 1050 • • • • • TOCTOCAGEA TOCTTOTOTO TTOTCTCTCT CTCACTGTGT TTCTGTATTT GTCTGAAAAT TAGGGCCAGA ACGACCTCCT ACCANGACAC AACAGAGACA GACTGACACA AAGACATAAA CAGACTTTTA ATCCCGGTCT >xoc1 >Saul XY21 >Kst2 ellalar >Bou36I >1/12 >2co411 • 1000 1100 • • CTOTTACCAC TOCCTTANGT TICACCTTAG GTCACTCCAA ACATGTOCAG CCCATOCCTC ACAACCAGTC EACANTGOTO AGGGAATTCA AACTGGAATC CAGTGACCTT TCTACAGCTC GCCTAGCCAG TCTTGGTCAG

>AspA1 >Zao1

SEa 1

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Table 6 (Cont'd)

>K4 2 >BetE2 >Pot1 |>Ball >X442 1170 • • 40 • • • • • • COTACATOTO AAGAACACAC GTTCCCTTAC CTTCTCCTCT GCAGAATCCC CAACCTTTAA CGTCCCATCG CCATCTACAG TICTTCTCTG CAACCCAATG GAAGACCAGA CGTCTTACCG GTTCGAAATI GCAGCCTACC >Ban1 >Hph1 >Hph1 1230 1240 **y•** • COCOCACAO GCACCITTAA COCAGACCTC ATCACCCACC TTAAGATCAA GGTCTTTTCA CCTCCCCCCC EGCECTETEC CETECAAATT EGCTETEGAG TAGTECETCE AATTETAGTT CEAGAAAAGT EGACCECCE >2001 >Dra2 • >EcoO1091 - 1 >Ppuki >7ch1111 >Styl • age Greener . ATGGACACCE AGACCAGGTE CECTACATOS TGACCTGGGA AGCCTTGGCT TTTGACCCCC CTCCCTGGGT TACCTUTGGG TETEGTOCAG GCGATGTAGC ACTGGACCCT TOCGAACCCA AAACTGGGGG GAGGGACCCA >Rsal 1370 • CAAGCCCTTT GTACACCCTA AGCCTCCGCC TCCTCTTCCT CCATCCGCCC CGTCTCTCCC CCTTGAACCT STTCCCCAAA CATGTCCCAT TCCCACGCCC ACCACAGGCA GGTACCCCCC GCACAGAGGC GCAACTTCCA • OCTOOTICEA OCCOCCOTOS ATOCTCOCTI TATCCAGOCC TCACTOCTTC TCTACOCCOC AATTCGTTAC CCACCAACCT CCCCCCCACC TACCACCCAA ATACCTCCCC ACTCACCAAC ACATCCCCCC TTAACCAATC

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Table 6 (Cont'd)
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    GAGTTGGGAC TGATAGAAGG TCCAGTAACA AGGTTGTACC GGGACACCTA GCTGTCCTAC GTTGAGGACA
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Table 6 (Cont'd)

>Xbal							>41,75
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ety1				>Ccrl			
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	1970	1980	1990	2000	2010	2020	2030
CCTAG	GGAC	TTARTCACC	141001100		• •	• •	
CGATC	CCIC	ANTINGTOGT	TATAGTTGCA	TATCALCAC	CACCTANAGE (CTCGATTTCC (CATCTGALAC	MCATTCATC
						-1404C111C	TIGTAGTAC
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			>Bet	21			nergee.
	2040	3010	***	**			ميندو در مديد در کار چي در مديد
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							, p
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							NVG# quantum.

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Table 6 (Cont'd)
                                                     >Hep(7524)1
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                                                        >HepH1
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                                                        >Sph1
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  TOTCAGTTAG GOTGTGGAA GTCCCCAGGG TCCCCAGCAG GCAGAAGTAT GCAAAGCATG CATCTCAATT
 ACAGTCAATC CCACACCTTT CAGGGGTCCC AGGGGTCCTC CCTCTTCATA CCTTTCCTAC GTAGAGTTAA
                                                              r office.
                                                           >We11 _...
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                                                          >Ecot22I
                                                           >Kep(7524)1
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                                                         >Xep#1
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               2260
              z270
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                        2270
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AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA TGCATCTCAA
TCACTCCTTC GTCCACACCT TTCACCCGTC CCACCCGTCC TCCCTCTTCA TACCTTTCCT ACCTAGAGTT
               2330
                      2340
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TTAGTCAGCA ACCATAGTOE OSCOCCTAAC TCOGCCCATC COCCCCCTAA CTCOGCCCAG TTCOGCCCAG
                                                            MATCHGTOGT TOGTATCHOG GOOGGGATTG ACCOCCGTAG GGOGGGGATT GAGGOGGGTA AAGGOGGGTA
     >Ncol
                                                 >8611
       •
     >5ty1
                                                 >3911
                                                  •
     2390
                                 2420
                                           2430
      4.
PETECGOCCO ATGGCTGACT AATTITTTT ATTIATGCAG AGGCCGAGGC CCCCTCGCC TCTCAGCTAT
ACAGGGGGGG TACCCACTGA TTALLLALA TALLTACCTC TCCCCCTCCC CCCCACCCC ACACTCCATA
                                                               .....
                           >5ty1
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                           >Ave2
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                          >8tu1
                                           >BepK1
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Table 6 (Cont'd) >Kind3 >Aat1 - 1 2500 2480 • 🕌 **v** • TOCACAAGTA GTCACCACCC TTTTTTCCAG GCCTACCCTT TTCCAALLAG CTTCCCCTCC ACGTCCACCC ACCTUTICAT CACTUCTOCO ANAMACCTO COCATUCCANA ANCETTITTO GARCECCACO TOCACCTOCO No. >Bcl1 >Iho? >BepK1 ag. >BatY1 lmian_virus_DHA_end/InS_DHA_etart 2570 2550 SCATCTCATC AAGAGACAGG ATGAGGATGG TITCGC ATG ATT GAA CAA GAT GGA TTG CAC GCA GGT TCT COTAGACIAG TICTOTOTO TACTCOTAGO MAGOS TAC TAA CIT GIT CIA CCT AAC CTO CCA AGA Het Ile Glu Gin Asp Gly Leu Bis Ala Gly Ser>

>Ecos21

>Eog1

>Coff1

>Inal

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>Hae2
>Bbel
>Harl
>Acyl
>Aha2

Table 6 (Cont'd)

>Ban1

2660 2670 268 | 2690 2700 2710

GAT GCC GCC GTC TTC GGG CTG TCA GCG GAG GGG GGC GGG GTT CTT TTT GTC AAG ACC GAC CTG
CTA CGG CGG CAC AAG GCC GAC AGT CGC GTC CCC GGG GGC CAA GAA AAA CAG TTG TGG CTG GAC
Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe Val Lys Thr Asp Leu>

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Table 6 (Cont'd)

							•	
2910	2920	2	930	2940	_	2950	3 2 2 60	
• •	• •	•	•	• •		co cc.		CNA COS
GCT GAT GCA	ATC CCC CC	O CTG CAT	ACC CTT	CAT COD	ect acc a	CC CCA	TTC GAC CAC	CTT CCC
CCA CTA CGT	TAC GCC GC	C GAC GTA	TCC CAA	CTA CCC	ble The C	ve Pro	ANG CTG GTG	Gln Ala>
Ala Asp Ala	Het yed y	ed ren wre	THE LEG	wab are	M.B 1	,	Phe Asp His	
		>80	a 1				.arq. ∉	
		>Hg LA1						
					r 10 I			
		>Xae2		~ 1	* F 101		j+ ÷	
	2980	2990	3	000	1 3010)	3020	3030
2970	•		•	•		•	•	• •
ANA CAT COS	ATC GAG O	CA GCA CGT	ACT CCC	ATG GAA	coc cet c	TT GTC	GAT CAG GAT	CAT CTG
	**** CTC G	CT CCT CCA	TCA GOC	TAC CTT	oce ocy e	YY CYG	CIA GIG CIA	CIX CAC
Lye Ris Arg	Ile Glu A	rg Ala Arg	The Arg	Het Glu	Ala Cly L	eu Val	Asp Gln Asp	Asp Leu>
•							. · = E	
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								•
							1	p(7524)1
							- 14.57	•
							>800 KZ	•
		>Ban2					>800 22	>NepH1
30	340	•	3060		3070	30		•
•	040 • •	3050	•	•	•	•	80 ATT 3	>KepH1
GAC GAA GAG		3050 •	ccy ecc	ead ctg	TTC GCC A	GG CTC	Ne cos osc	>HepH1
CAC CAA CAA	CAT CAG G	3050 CC CTC CCC	CCA GCC	CTT GAC	TTC GCC A	AGG CTC	ANG GOO GOC TTC GOC GOC	>HepH1
CAC CAA CAA	CAT CAG G	3050 CC CTC CCC	CCA GCC	CTT GAC	TTC GCC A	AGG CTC	Ne cos osc	>HepH1
CAC CAA CAA	CAT CAG G	3050 CC CTC CCC	CCA GCC	CTT GAC	TTC GCC A	AGG CTC	ANG GOO GOC TTC GOC GOC	>HepH1
GAC GAA GAG CTG CTT CTG Asp Glu Glo	CAT CAG G	JOSO CC CTC GCC CC GAG CCC Ly Leu Ale	CCA GCC GCT CCG Pro Ala	CTT GAC	TTC GCC A	AGG CTC	ANG GOO GOC TTC GOC GOC	>HepH1
GAC GAA GAG CTG CTT CTG Asp Glu Glo	CAT CAG G	3050 CC CTC CCC	CCA GCC GCT CCG Pro Ala	CTT GAC	TTC GCC A	AGG CTC	ANG GOO GOC TTC GOC GOC	>NepH1 090 ATG CCC TAC GGG Net Pro>
CAC GAA GAO CTG CTT CTO Asp Glu Glo	CAT CAG G CAT CTC G Mie Gin G	JOSO CC CTC GCC CC GAG CCC Ly Leu Ale	CCA GCC GCT CCG Pro Ala	CTT GAC	TTC GCC A	AGG CTC	ANG GOO GOC TTC GOC GOC	>NepH1 090 ATG CCC TAC GGG Net Pro>
CAC GAA GAO CTG CTT CTO Asp Glu Glo	CAT CAG G	JOSO CG CTC GCC CC GAG CCC Cly Leu Ale	CCA GCC GCT CCG Pro Ala	CTT GAC	TTC GCC A	AGG CTC	ANG GOO GOC TTC GOC GOC	>NepH1 090 ATG CCC TAC GGG Net Pro>
CAC GAA GAO CTG CTT CTO Asp Glu Glo	CAT CAG G CAT CTC G Mie Gin G	JOSO CG CTC GCC CC GAG CCC Cly Lau Ala >No	CCA GCC GCT CCG Pro Ala	CTT GAC	TTC GCC A AAG CGG T Phe Ale A	AGG CTC	ANG GOO GOC TTC GOC GOC	>NepH1 090 ATG CCC TAC GGG Net Pro>
GAC GAA GAA CTG CTT CTA Asp Glu Gla >X >B	CAT CAG G CTA GTC G NIO GIA G	JOSO CCC CAC CCC CLY Law Ala >No	CCA GCC GGT CCG Pro Ala	CTT GAC Glu Leu	TTC GCC A AAG CGG T Phe Ale A	DIAO	AAG GCG CGC TTC CGC GCC Lyo Ala Arg	>HepH1 OTO ATC CCC TAC GGG Het Pro> >Cfr1 >Lae1
CAC GAA GAA CTG CTT CTA Asp Glu Gla >X >B	CAT CAG G CTA GTC G NIO GIA G	JOSO CCC CAC CCC LAU ALA >NO >ST	CCA GCC GGT CCG Pro Ala	CTT GAC Glu Leu J13 GAT GCC	TTC GCC A AAG CGG T Phe Ale A	JI40	AAG GCC GCC TTC CCC GCC Lyo Ale Arg	>HepHi OTO ATC CCC TAC GGG Het Pro> >Cfri >tael
GAC GGA GAC GTG CTT CTC Asp Glu Glu >X >B 3100 GAC GGC GA	CAT CAC C COTA GTC C COTA GTC C COTA GTC C COTA GTC C COTA GAC CTC C	JOSO CCC CAC CCC LAU ALA >HO CCC CAC CCC CLY LAU ALA CCC CAC CCC CLY LAU ALA CCC CAC CCC CCC	CCA GCC GGT CCG Pro Ala 101 1120 CAT GCC GGTA CCC	CTT GAC Glu Leu J13 GAT GCC CTA CCG	TTC GCC AAAG CGG TAAAG CGG TAAAG CGG TAAAG CGG TAAAG CAAAAG AAAC AAAAG AAAA AAAA	JI40	AAG GCG CGC TTC CGC GCC Lyo Ala Arg	>Hephi OTO ATC CCC TAC GGG Het Pro> >Cfri >tael GAA AAT CTT TTA

Table 6 (Cont'd) >Cfr10I ×(trl >Rer2 >Eael >Hael 3210 3200 3180 3170 3160 GGC GGC TIT TOT GGA TTC ATC GAC TGT GGC GGG CTC GGT GTC GGG GAC GGC TAT CAG GAC ATA COS GOG ANA AGA COT ANG THE CTG ACA COO GOC GAC COA CAC CGC CTG GOG ATA GTC CTG TAT Gly Arg Phe Ser Gly Phe Ile Asp Cye Gly Arg Lou Gly Val Ala Asp Arg Tyr. Gla Asp Ile> 3280 3260 3250 3230 3220 SOO TIG SOT ACC OCT GAT ATT SOT GAA GAG CTT SOC SGC GAA TOS SOT GAC OCC. TTC CTC STC COC AAC COA TOG GCA CTA TAA COA CTT CTC GAA CCC CCC CTT ACC CCA CTG GCC AAG GAG CAC Ale Leu Ale Thr Arg Asp Ile Ale Glu Glu Leu Gly Gly Gle Trp Ale Asp Arg Phe Leu Val> 3330 3320 3310 3290 CTT THE GGT ATE GGE GGT CCC GAT TOO CAG CGC ATE GGC TTE THE CGC CTT CTT GAC GAG TTE GAA ATG CCA TAG OCC OCA GGG CTA AGC GTC GOC TAG OCC AAG ATA GOC GAA GAA CTC CTC AAG Lou Tyr Gly Ile Ala Ala Pro Asp Ser Gla Arg Ile Ala Phe Tyr Arg Leu Leu Asp Glu Phe> >Ple1 >TnS_DXA_end/_No-NuLV_DXA_start 3390 3370 3350 . TTC TCA GOOGGACTC TOGGGTTOCK TAUNTAUN CATTTTATTT AGTCTCCACA ANNOGGGGG ANTCANGAC AAG ACT OGCOCTGAG ACCOCCUGCT ATTTATTTT CTANATANA TCAGAGGTCT TTTTCCCCCCC TTACTTTCTC Phe End> >Af12

3430 COCACCTOTA GGITTGGCAA GCIAGCTTAA GTAACGCCAT TTTGCAAGGC ATGGAAAAT ACATAACTGA OCCIOCACAT CCAAACCCIT CCATCCAAIT CATICCCCTA AAACCITCCC TACCITITIA TOTATICACT

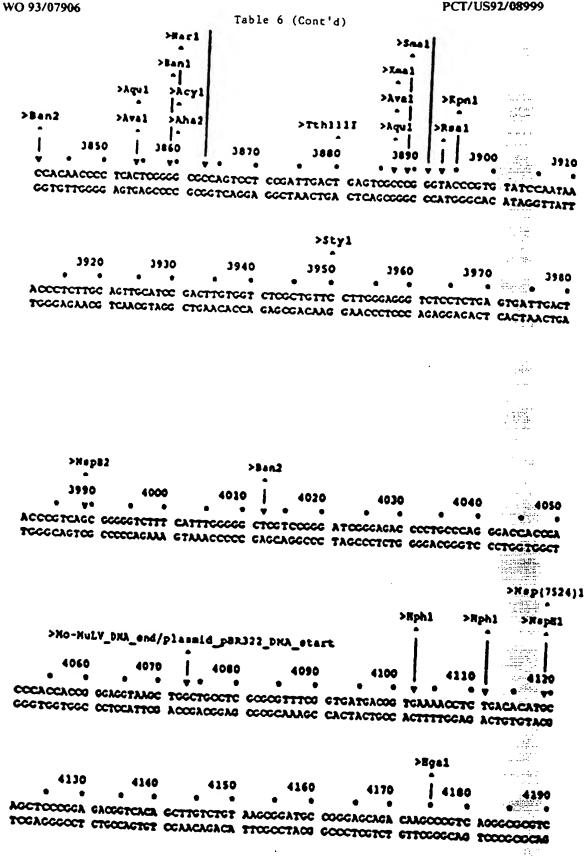
>NepB2 >ZcoZS Surt< GRATAGAGAA GTTCAGATCA AGGTCAGGAA CAGATGGAAC AGGTGAATAT GGGGCAAACA GGATATGTGT CITATCICIT CAACICIACI TOCACICCIT GICTACCITG TOCACITATA COCCUTION CETATAGACA WO 93/07906

PCT/US92/08999

>Nap82

>AlwKl >Pvu2 >EcoRS 3590 3600 3610 3630 ** • GGTAAGCAGT TCCTGCCCCC GCTCAGGGCC AAGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA CCATTCGTCA AGGACGGGC CGAGTCCCGG TTCTTGTCTA CCTTGTCGAC TTATACCCGG TTTGTCCTAT >Alwn1 3670 3680 • TOTOTOGTAN GENGTTCCTG COCCOCCTCN COCCCNNON CNGNTGGTCC CENGNTGCG TCCNCCCCTC AGACACCATT COTCAAGGAC GGGCCCAGT CCCGGTTCTT GTCTACCAGG GGTCTACGCC AGGTCCCGAE >Pesl >Eco01091 >Xbal >Banl • • 70 • V. V . • AMBIEL 0 AGCAGITTET AGAGAACCAT CAGATEITIC CAGGGICCCC CAAGGACCTG AAATGACCCT GTGCCTTATT POSTCAAAGA TETETTEGTA GTETACIAGI GTECCACOGG GTTECTGGAC TITACTGGGA CACCGAATAA >Saci • >BqLL1 >Set1 >Banl TGAACTAACC AATCAOTTCO CTTCTCCCTT CTCTTCCCCC GCCTCTCTCCTC CCCCAOCTCA ATAAAACACC actigating traticalse embresses exclusives occasione ecceptocage extitutives >Banl >Asp718 >Bbel

PCT/US92/08999



>7th1111

>Kae2

>Acc1

>Nep82

61 PCT/US92/08999 Table 6 (Cont'd) 4200 4210 4220 4230 | 4250 4260 • • • • **v** • • AGCCCCCTCTT GCCCCCTCTC GCCCCCCACC CATCACCCAC TCACCTAGCC ATAGCCCACT GTATACTCGC TOGCCCACAA COCCCCACAG COCCCCTCG GTACTGGGTC AGTGCATGGC TATCGCCTCA CATATGACCG >Hg LA1 >Real >Apall >Ndel 14300 4320 4330 • • • • *** • •** TTANCTATEC GECATCAGAG CAGATTETAC TEAGAGTECA CEATATECEG TETGAAATAC CECACAGATE AATTGATAGG COGTAGTCTC GTCTAACATG ACTCTCAGGT GGTATAGGCC ACACTTTATG GOGTGTCTAG >H402 >Ple1

4360 4380 *** •** • • **v•** • • • CETANGGAGA ANATACCICA TCACCOCCTC TTCCCCTTCC TCCCTCACTC ACTCCCTGCC CTCCCTCCTT GCATTCCTCT TITATCGCCT AGTCCCCCCAC AACGCCAAGG AGCCACTCAC TCAGCCACCC GAGCCACCAA

4430 4440 • • • • • • • • • OCCUPACION DE LA CONTRACTOR ANGECCETAR TACCETTATE CACAGARTER GEOGRAFANCE GCCCACCCCC CTCCCCATAG TOCAGTCAGT TTCCCCCCATT ATGCCAATAG GTGTCTTAGT CCCCTATTGC

1- 2 288E.

- HESSE.

11.63 7

>Map(7524)1 >Map#1 >113 4480 4510 4530 CACCALAGAA CATCTCACCA ALACCCCACC ALAACCCCAG GLACCCTALA AACCCCCCT TOCTCGCCTT efectificit etacactori ittoccetor itticoccio citoccatti itoccecocca accaccoccaa

>Hgal • 4550 4560 4570 4580 4600 4610

Table 6 (Cont'd) TITICATAGG CTCCCCCCCC CTCACCACCA TCACAAAAAT CCACCCTCAA CTCACACCTC GCCAAACCCC AAAGGTATCC GACGCCCCC GACTGCTCGT AGTGTTTTTA GCTGCGAGTT CAGTCTCCAC CGCTTTCGGC 4680 . ACAGGACTAT ALAGATACCA GGCGTTTCCC CCTCGAAGCT CCCTCGTGCC CTCTCCTGTT CCGACCCTGC . TOTOCTCATA TITICTATGGT COCCUAAGGG GCACCTTOGA GGGAGCACGC GAGAGGACAA GGCTGGGACG >Rae2 4730 OCCUTAÇÕES ATACCTUTOS GOCTITUTOS CITOGOGAAS OCTOGOCCTI TOTOATAGOT CAOGCTOTAG COMMITTEE TATEGRACIOS COMMITTES GARGOCTTO GOACOGOMA AGAGTATOM GTOCCACATO >HQLA1 >ApaL1 4800 GIATCTCACT TECCTCIACE TECTTOCCTC CAACCTCCCC TETCTCCACE AACCCCCCAC TCACCCCCAC CATAGRATICA AGCERCATOE AGENAGOGAG GTTCGACCOG ACACACOTOC TTCCCCCCCA AGTCCCCCTC >Nep82 >2101 4860 . OCCIGOCOCT TATEOCOTAL CTATOCTOTT GAGTOCAACC COCTAAGACA CGACTTATOC CCACTGGCAG GOCACCCCCA ATACCCCATT GATACCACAA CTCACCTTCG GCCATTCTGT CCTCAATACC GGTGACCCTC >Alwk1 • CAGCCACTEG TAACAGGAT AGCAGAGGA GGTATGTAGG CGGTGCTACA CAGTTCTTGA AGTGGTGGGC STOCCTORCC ATTETECTIA TOUTCTOCT CCATACATEC GCCACCATET CTCAGACT TCACCACCGG

4970 4980 4990 5000 5010 5020 5030
TAACTACGCC TACACTAGAA GGACACTATT TGGTATCTGC GCTCTGCTGA AGCCAGTTAG CTTCGGAAAA
ATTCATGCCG ATGTGATCTT CCTGTCATAA ACCATAGACC CGAGACGACT TCGGTCAAG GAAGCCTTTT

>Neps2

WO 93/07906

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PCT/US92/08999
                         Table 6 (Gont'd)
                                $070 $080 $090 ........ $100
                        • •
  ACACTTECTA CCTCTTCATE CCCCALACAA ACCACCECTE CTAGEGGTGG TITTTTTGTT TECAAGCAGE
  TCTCAACCAT CGAGAACTAG GCCCTTTGTT TCGTGGCCAC CATCGCCACC AAAAAACAA ACGTTCGTCC
                   >Xho2
                            >BetTl
                                                          >#gal
                   >BetY1
                            >Iho2
                                                          5170
                               5140
                                          5150
                                                    5160
                                                  • • ....•. 70
               •
  AGATTAGGGE CAGALAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT AGGGGGTCTG AGGTCAGTG
  TCTAATGCCC GTCTTTTTT CCTAGAGTTC TTCTAGGAAA CTAGAAAAGA TGCCCCAGAC TGCCAGTCAC
                                          >BetYl
                                                        >BetTl >Dral
                                         >Xho2
           >Xae2
                          >8epH1
                                        >Hph1
                                                     >Xho2 -------->Ahe3
                                        - 1
                                                         5240
                         5200
                                         |5220
                                        ,-464
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 CAACCALLAC TCACCTTAAG GCATTTTGGT CATCACATTA TCAALAAGGA TCTTCACCTA GATCCTTTTA
 CTTCCTTTTC AGTCCAATTC CCTAUACCA GTACTCTAAT AGTTTTTCCT AGAAGTCGAT CTACGAUAT
          T# >Dral
                                                          .
               $260
                         5270
              . . .
                       • •
                               •
                                        •
 ANTIQUANT CANCETTIAN ATCUSTEN ACTATATATE ACTANACTIC CTCTCACACT TACCALISECT
 TEMPETERS CETCALLATE PAGETAGATE TOATATAGE TOATTEGASC CAGACTOTCA ATCOFFACEA
         >Banl
                                             >Ple1
                                $350
                       • •
                               • •
                                        . . . .
 TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTAFFTCO TTCATCCATA GTTGCCTGAC TCCCCGTGCT
 ATTACTCACT COCTOCATAG ACTOCCTACA CACATALACC AACTACCTAT CAAOCCACTG ACCOCCACCA
                                                       >8041
                                3420
                                         5430
                              • •
                      • •
                                        • •
 CATCHATTER TECTATECCE TECCERATES TREACCISES TERCEACETT ACTATECCES TETECETICS
>Cfr101
                                   >3g11
               5470
                              5490
                        5480
             . . . .
                                                        .....
TCACCCCCTC CAGATTTATC AGCALTARAC CAGCCAGCCC GAAGGCCCCCA GCCCAGAAGT GCTCCTGCAA
ACTOCCOCAC CTCTAAATAG TOCTTATTTC CTCCCTCCC CTTCCCCCCCT CCCCTCTTCA CCACCACCTT
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Table 6 (Cont'd)

						9171- No.701 1.077-
		>Asel				e in the second
5530	\$\$40			\$\$70	5580	359
CTTTATCOGC	CTCCATCCAG	TCTATTAATT		• •	AGTAGTTOGC	CAGTTAATA
GAAATAGG∝						
						1212.1
>Hae	2					4 5 5 10 5 1 5 7
>1001						¥
>Fep1						-
-						* ***
>Fd12						grafic.
>Hetl		>Pet1				. j. <u>j. j. j.</u> 1. j. j. j.
5600	5610			• • • •	5650	5660
TTTGCCCAAC	GTTGTTGCCA	•				- 特別的は最高の対象
MACCOTTC				_		
						() () () () () () () () () ()
5670	5680	\$690	-	\$710	_	\$730
AGCTCCGGTT	CCCAACCATC					GTTAGCTCCT
TOCAGGCCAA	COGTTCCTAG	TTCCCCTCAA	TGTACTAGGG	GGTACAACAC	GITTITICGC	CAATCGAGGA
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To generate the LXSN-RI-IL2 retroviral vector, 10 micrograms of pLXSN-RI-IL2 DNA was transfected into the ecotropic packaging cell line PE501 by standard calcium phosphate precipitation methods (Miller et al., Mol. Cell 5 Biol. 6:2895, 1986). The transfected PE501 cell line was grown in DMEM medium with 10% FCS. The medium was changed after 24 hours and supernatant harvested 24 hours later to infect the amphotropic packaging cell line PA317 as described (Miller et al., Mol. Cell Biol. 6:2895, 1986 and 10 Miller et al., BioTechniques 7:980, 1989). The infected PA317 cells were harvested by trypsinization 24 hours later and replated 1:20 in DMEM containing 10% FCS and the neomycin analogue G418 (400 μ g/ml). The cells were grown at 37°C in 7% CO₂ atmosphere. The selection medium was 15 changed every 5 days until colonies appeared. On day 14, twenty colonies were selected, expanded and tested for viral production by standard methods (Xu et al., Virology 171:331-341, 1989). Briefly, supernatants were harvested from confluent culture dishes, passed through a .45 μm 20 filter, diluted with DMEM with 10% FCS and utilized to infect NIH 3T3 cells in the presence of 8 μ g/ml polybrene. After 24 hours, the infected NIH 3T3 cells were grown in culture medium that contained the neomycin analogue G418. After 12-14 days, the colonies were stained, counted and 25 the viral titer calculated as described (Xu et al., Virology 171:331-341, 1989).

Colonies with the highest viral titers (>10' infectious units/ml) were tested for IL-2 expression by Northern blot analyses. Colonies with the highest viral titers and documented IL-2 expression were cryopreserved and will be utilized as stock cultures to produce the LXSN-RI-IL2 retroviral vector trial.

EXAMPLE IV

RETROVIRAL VECTOR CONSTRUCTION AND CYTOKINE EXPRESSION

lines, vectors were used containing different promoters to drive IL-2 expression, and a human IL-2 cDNA was directionally sub-cloned into the insulin secretory signal peptide (17). The IL-2 cDNA was directionally sub-cloned into the parental plasmids of the LXSN (LTR promoter) and LNCX (CMV promoter) vectors (gifts of Dr. A.D. Miller) (18). The newly constructed vectors (Figure 1), designated as LXSN-IL2 and LNCX-IL2, were packaged in the PA317 cell line for production of retroviral supernatant. As a control, the high level expressing, double copy vector DC/TKIL-2 vector (thymidine kinase promoter) (a gift of Dr. 15 E. Gilboa) was used for comparison.

These vectors were used to transduce a number of murine and human, primary and established cell lines. Pools of transduced cells were selected and expanded in DMEM medium, containing 10% fetal bovine serum (FBS) and 400 µg/ml of active G-418, a neomycin analogue. The results of expression studies in the MCR9 and Balb/c 3T3 cell lines are presented in Table 7.

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Table 7

Comparison of	IL-2	expression by	fibroblasts
transduced	with	different IL-	vectors.

5		 				11111	
			ng I	<u>L-2</u>	<u>-2 Units IL-2</u>		
	Fibroblast	Vector	per	10° cells	per day		
	Murine	LNCX (Contro	0.4	±50%	<1	و المارية	
10		LNCX-IL2	33.7	±11%	67	+ ##T. T	
		LXSN-IL2	6.6	± 6%	13		
		DC/TKIL-2	1.9	± 5%	4		
	Human	LXSN (Contro	1) 0.7	±29%	1	Tana da Caranta	
		LNCX-IL2	159.5		319	+ +	
L 5		LXSN-IL2	25.5	±15%	51	AND	
		DC/TKIL-2	3.0	±10%	6	i i i i i i i i i i i i i i i i i i i	

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EXAMPLE V

FIBROBLAST CULTURE AND CONDITIONS FOR RETROVIRAL TRANSDUCTION

The culture conditions for the growth of primary fibroblasts retroviral transduction were optimized. Primary fibroblasts were successfully cultured. The optimal conditions enable the growth of approximately 3-4 x 10° primary fibroblasts from a 12 mm² skin biopsy in approximately 4-6 weeks. Retroviral infection, G418 selection, and expansion of the genetically modified fibroblasts takes an additional 4-6 weeks.

Exploring the conditions for genetic modification of primary fibroblasts suggests that optimal transduction may be obtained by the following procedure: The fibroblasts are synchronized in Gl phase by serum starvation, followed by stimulation with medium containing 15% fetal bowine serum 15 hours prior to transduction. The cells are then subjected to 2 cycles of retrovirus infection, each cycle lasting approximately 3 hours. The cells are refed with fresh media overnight, and then selection in G418 is initiated the next day. This method is capable of transducing 5-15% of the fibroblasts in a culture, depending on the multiplicity of infection.

This procedure was used to transduce a large number of primary and established fibroblasts. As an example, Table 8 compares the expression levels of IL-2 in fibroblast lines transduced with LXSN-IL2.

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	0 1				
Expression of	t IL-2 by	'Ilbroblasts	transduced	with	LXSN-IL2.

5	Fibroblast]	ng IL-2 Unit	s IL-2
	Line	Species	Origin pe	r 10° cells p	per day
	Balb/c 3T3	Murine	Transformed	6.6 ± 6%	13
	MCR9	Human	Embryonic	25.5 ±15%	51
0	NHDF 313	Human	Skin	25.0 ±10%	50
	GT1	Human	Skin	15.0 ± 5%	30

These results indicate that the IL-2 expression levels in established, embryonic, and primary fibroblast cultures are similar. Comparison of these data with Table 7 suggest that IL-2 expression is affected more by factors such as different promoters than by the fibroblast line used. Similarly, changes in culture conditions can have important effects on IL-2 expression. Table 9 shows that transduced GTl cells, a primary human fibroblast culture expressed 15-fold more IL-2 under 100 µg/ml G418 selection than under 25 µg/ml G418 selection. Several other primary fibroblast lines have also been transduced with our vectors and are currently growing under G418 selection.

Table 9

Effect of	G418 concentration on IL-2 expression b	oy GT1
	cells transduced with LXSN-IL2.	:

5					
•	Selection dose	ng I	L-2	secre	ted
	of G418	per 106	ce	lls pe	r day*
•	25 μg/ml	1.0	±	10%	::Leiche + 1727 (201)
10	50 μ g/ml	3.0	±	6%	orization Seedings States
	100 μ g/ml	15.0	±	5%	. 195

*After three weeks of G418 selection.

EXAMPLE VI

COMPARISON OF IL-2 EXPRESSION LEVELS INDUCED PERIPHERAL BLOOD LYMPHOCYTES AND GENETICALLY MODIFIED FIBROBLASTS

In order to compare the production of IL-2 by genetically modified fibroblasts to that achieved by stimulating normal human peripheral blood lymphocytes 20 (nPBL) in vitro, nPBL were isolated by Ficol-Paque density centrifugation, and cultured in the presence of allogeneic nPBL (mixed lymphocyte culture, MLC) or 2 µM calcium ionophore (CI) (A23187) free acid) plus 17 nM phorbol 12-25 myristate 13-acetate (PMA). The results of this experiment, present in Table 10, indicate that the level of IL-2 expression in the PMA/CI stimulated normal T cell population was 2 ng/10' cells/24 hours. This is equivalent to IL-2 expression by Balb/c 3T3 fibroblasts transduced 30 with DC/TKIL-2 (Table 7), our least productive vector. The level of IL-2 expression in the MLC was 130 pg/106 cells/24 hours. This was lower than the PMA/CI stimulated culture, presumably because PMA/CI induced a nonspecific response

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while MLC resulted in specific Th stimulation. When the estimated percentage of antigen-specific Th in the MLC-stimulated population is tak n into consideration, the level of IL-2 expression per stimulated T cell becomes equivalent for both methods.

Table 10 Levels of IL-2 secretion by different cells.

10	Cells	pg IL- per 10° c		se crete d ls per day
	Lymphocytes:			1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
	Control (non-activated)	5	±	50%
	PMA + Calcium Ionophore	2,000	±	68
15	Mixed lymphocyte culture	130	±	90%
	Transduced fibroblasts:			
	MCR9-LXSN-IL2	24,000	±	58
	MCR9-LNCX-IL2	162,000	±	20%
	MCR9-DC/TKIL-2	10,000	±	68
20				To Marketon

EXAMPLE VII

FIBROBLAST MEDIATED CYTOKINE GENE THERAPY IN MURINE TUMOR MODELS

efficacy of fibroblast-mediated cytokine gene therapy on induction of anti-tumor immunity. The first protocol was designed to test the effects of genetically modified fibroblasts on tumor implantation, while the second protocol was designed to induce a systemic anti-tumor immunity. The results of each experiment are presented with two figures and one table. In the first figure, the rate of tumor growth for each treatment group is pres nted

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as the mean tumor size in the group over time. In the second figure, a Kaplan-Meier curve presents the time of tumor onset for the individual animals in each tratment group. The number of animals, the number and percentage of tumor free animals, and the tumor size distribution patterns for each experiment are presented in a table.

EXAMPLE VII(a)

EFFECT OF FIBROBLAST MEDIATED CYTOKINE GENE THERAPY ON TUMOR IMPLANTATION

Mice were injected subcutaneously with mixtures of 5 x 10° CT26 cells and 2 x 10° fibroblasts genetically modified by different retroviral vectors to express IL-2. In the control arms injected with tumor cells only, or with tumor cells mixed with unmodified fibroblasts, 31 of 33 animals (94%) developed tumors by 4 weeks (Figures 6 and 7, Table 9). In contrast, 22 out of the 34 animals (65%) receiving fibroblast mediated cytokine gene therapy were tumor free at 3 weeks, and 5 animals (18%) remain tumor free after 12 weeks. Those animals that received fibroblast mediated IL-2 therapy and developed tumor were characterized by a delayed onset and rate of tumor growth.

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Table 11

Effect of IL-2 modified fibroblasts on tumor establishment and development. 2 X 106 fibroblasts mixed with 5 X 104 CT26 tumor cells at time of injection.

Gibrahleete	∢	Tumor-	Animal Number Tumor- Tumor-	Percent		Tumor Si	fumor Size (mm²)		Median Tumor Size	Tum	r Size
mixed with tumor cells	Total	<u>8</u>	bearing	Tumor-free	25-100	101-200	25-100 101-200 201-300 > 301	> 301	<u>ت</u>	Em ²	
A Acc 12 Washer							•	-			
DIEL 12 HONE	;	•	;	80	_	c	_	•	420 + 145	+	145
Control (no fibroblasts)		•	=	£	-	>	•	•		ł	
Unmodified fibroblasts**	13	7	=	15%	-	0	****	_	388	#1	265
DOTEK-II 2 filmblasts	13	0	<u>E</u>	% 0		6	S	4	267	#	168
1 NCX-II 2 fibroblasts	51	8	60	39%	'n	7	0		22	+1	8
	,										

Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

Two mice in this arm developed intraperitoneal tumors which were not measurable.

After 3 weeks the mean tumor size (measured as the product of the longest and widest tumor axes) in the control group of mice was 128 mm², compared to 68 and mm² in groups of mice injected with tumor cells mixed with 5 fibroblasts transduced with DC/TKIL-2 LNCX-IL2. respectively. This resulted in a highly significant difference (corrected $x^2 = 18.69$, p = 0.001) between the IL-2 treated animals compared to the mice treated with CT26 alone or CT26 mixed with unmodified fibroblasts. After 10 four weeks the equivalent measurements were 373,300 and 72 mm2 (Table 11). It is notable that LNCX-IL2, the highest expressing vector caused substantially greater inhibition of tumorigenicity than the lower expressing vector DC/TKIL-A multivariate non-parametric statistical procedure (19,20), utilized to evaluate differences in tumor growth, demonstrated that after 4 weeks the differences between the growth curves for the four groups presented in Figure 2 were highly significant (p < 0.001). Subsequent comparisons between the control arm and animals that 20 received tumor cells mixed with IL-2 transduced fibroblasts revealed a significant difference (P < 0.05). The differences between the animals injected with tumor cells alone, and those injected with tumor cells plus unmodified fibroblasts were not significant, while the differences 25 between animals receiving low IL-2 expressing fibroblast, and those receiving high IL-2 expressing fibroblasts was significant (P = 0.05).

When mice were injected with 2 x 10° modified fibroblasts mixed with 1 x 105 live tumor cells the results 30 became more striking (see Figures 8 and 9, and Table 12). All the control animals developed tumors after 4 weeks whereas 33% and 27% of the animals treated with fibroblasts modified with the DCTK-IL2 or LXSN-IL2 vectors (respectively) remain tumor free after 7 weeks (the 35 experiment is ongoing). More dramatically, 75% of the animals treated with fibroblasts modified with th highest

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IL-2 producing vector, LNCX-IL2, remain tumor free after 7 weeks. These data clearly demonstrate the importance of an initial high dose of IL-2 to prevent tumor establishment.

Table 12

Effect of IL-2 modified fibroblasts on tumor establishment and development. 2 X 106 fibroblasts mixed with 1 X 105 CT26 tumor cells at time of injection.

Fibroblasts mixed with tumor cells	Total	Animal Number Tumor- Tum free beari	nber Tumor- bearing	Animal Number Tumor- Tumor- Percent Total free bearing Tumor-free	25-100	Tumor Size (mm²) 25-100 101-200 201-300 >301	.е (mm²) 201-300	> 301	Mean Tumor Size (mm²)	2 2
After 6 Weeks: *										
Control (no fibroblasts)**	13	0	13	% 0	0	8	7	S	315 ± 197	7
Unmodified fibroblasts**	70	0	20	80	0	2	е	7	350 ± 100	0
DCTK-IL2 fibroblasts	13	4	60	33%	0	-	4	ю	185 ± 141	_
LXSN-IL2 fibroblasts***	15	₹	=	27%	0	S	-	7	135 ± 121	
LNCX-IL2 fibroblasts	∞	9	2	75%	7	0	0	0	8 + 14	4

Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

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^{••} One mouse in each of these arms developed an intraperitoneal tumor which was not measurable.

Three mice in this arm developed intraperitoneal tumors which were not measurable.

As an additional control, mice were inject d with CT26 cells genetically modified to express IL-2 (results Injection of up to 1 x 106 IL-2 expressing not shown). tumor cells into Balb/c mice failed to produce tumors. 5 Injection of higher numbers however, resulted in some animals developing tumors with delayed onset. These data confirm the results reported in the literature (1). In order to compare the efficacy of IL-2 producing fibroblasts to IL-2 producing tumor cells, we mixed 2 x 106 CT26 tumor 10 cells modified with the DCTK-IL2 vector with 1 x 105 unmodified tumor cells. Figures 10 and 11, and Table 13 show that DCTK-IL2 modified tumor cells are somewhat Four weeks effective in preventing tumor development. after injection, the mean tumor size for the treatment arm is 303 mm2, compared to 620 mm2 for the control arm. After 22 weeks, one animal (10%) remains tumor free, compared to none in the control arms. Data for animals treated under the same conditions with DCTK-IL2 modified fibroblasts in a separate experiment are included for comparison purposes. 20 This comparison suggests that DCTK-IL2 modified tumor cells have an effect on tumor establishment similar to that of DCTK-IL2 modified fibroblasts.

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Table 13

Effect of IL-2 modified cells on tumor establishment and development.

2 X 106 DCTK-IL2-modified CT26 tumor cells mixed with 1 X 105 CT26 cells compared to 2 X 106 DCTK-IL2-modified fibroblasts mixed with 1 X 105 CT26.

	V.	Animal Number	nber						
Cells mixed with tumor cells	Total	Tumor- Fotal free	Tumor- bearing	Tumor- Tumor- Percent free bearing Tumor-free	25-100	Tumor Si 101-200	Tumor Size (mm²) 25-100 101-200 201-300 > 301	> 301	Mean Tumor Size (mm²)
After 22 Weeks:									
Control (no fibroblasts)	~	0	'n	80	•	0	0	S	620 ± 190
Unmodified fibroblasts	8	0	8	%	0	0	0	S	587 ± 69
DCTK-IL2-modifed CT26 cells	0	-	٥	10%	-	0	2	8	303 ± 179
DCTK-IL2-modified fibroblasts	cc	7	v	25%	0	-	7	m	214 ± 158

Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

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EXAMPLE VII(b)

EFFECT OF FIBROBLAST MEDIATE CYTOKINE GENE THERAPY ON SYSTEMIC ANTI-TUMOR IMMUNITY

Groups of Balb/c mice were immunized with 2.5 x 10⁵ irradiated tumor cells either alone or mixed with 2 x 10⁶ transduced or unmodified fibroblasts, and challenged one week later with 5 x 10⁴ live tumor cells in the opposite flank. These results (Figures 12 and 13, and Table 14) demonstrate that immunization with irradiated tumor cells and transduced fibroblasts protect some animals against a live tumor challenge, but that the protection is only slightly better than that achieved by immunization with irradiated tumor cells alone or irradiated tumor cells mixed with unmodified fibroblasts.

Table 14

Effect of IL-2 modified fibroblasts on induction of sytemic anti-tumor immunity.

Mice immunized with 2 X 106 fibroblasts mixed with 2.5 X 109 irradiated CT26 tumor cells 7 days prior to challenge with 5 X 104 fresh tumor cells.

	Ø.	nimel Nu	nber						
Fibroblasts mixed with irradiated tumor cells	Total	Tumor- free	Tumor- bearing	Tumor- Tumor- Percent otal free bearing Tumor-free	25-100	Tumor Si 101-200	Tumor Size (mm²) 25-100 101-200 201-300 >301	> 301	Mean Tumor Size (mm²)
After 22 Wæks:*									
Control (saline)	70	0	20	%	•	0		61	574 ± 160
Irradiated CT26 only**	16	~	=	31%	7	-	7	S	250 ± 277
Irradiated CT26 mixed with unmodified fibroblasts	15	4	=	27%	•	-	E	7	266 ± 199
DCTK-IL2 fibroblasts**	23	0	15	40%	4	-	-	80	172 ± 194

Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

•• One mouse in each of these arms developed an intraperitoneal tumor which was not measurable.

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In a second protocol similar to the one described above, animals were challenged with fresh tumor cells two weeks following immunization with irradiated tumor cells mixed with fibroblasts. The results, shown in Figures 14 5 and 15, and in Table 15, demonstrate that DCTK-IL2 modified fibroblasts mixed with irradiated tumor cells confers superior protection to subsequent tumor challenge than irradiated tumor cells alone, irradiated tumor cells mixed with unmodified fibroblasts, or irradiated tumor cells 10 mixed with LNCX-modified fibroblasts. After 7 weeks, seven of ten animals (70%) treated with DCTK-IL2 modified fibroblasts remain tumor free compared to only one third of the control animals. At four weeks, the mean tumor size of this group was 41 mm², compared to 180, 170, and 140 mm² for 15 the three control groups. Animals treated with LNCX-IL2 modified fibroblasts were also protected against subsequent tumor challenge, but the results were less striking. In this group, 54% of the animals remain tumor free and the mean tumor size for the group at four weeks was 86 mm2. The 20 number of tumor free animals in the group treated with LXSN-IL2 modified fibroblasts was similar to the control groups, although the tumors were slightly delayed in their onset. A multivariate non-parametric statistical procedure (19, 20), utilized to evaluate differences in tumor onset, 25 demonstrated that the differences for the six arms presented in Figure 15 were significant (p = 0.012). It further showed that the saline control arm and the arms that received irradiated tumor cells alone or mixed with unmodified or LNCX vector modified fibroblasts formed a 30 statistical group. A second, distinct statistical group was formed by the three arms that received IL-2 vector modified fibroblasts mixed with irradiated tumor cells. Subsequent comparisons between the saline injected control arm and animals that received tumor cells mixed with IL2 35 transduced fibroblasts revealed a significant difference for all vectors (p < 0.05).

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Table 15

Effect of IL-2 modified fibroblasts on induction of sytemic anti-tumor immunity.

Mice immunized with 2 X 106 fibroblasts mixed with 2.5 X 109 irradiated CT26 tumor cells 14 days prior to challenge with 5 X 104 fresh rumor cells.

Immunization by	4	Animal Number	절						
fibroblasts mixed with irradiated tumor cells	Total free	Furmor- free	Tumor- Tumor- free bearing	Percent Tumor-free	25-100	Tumor Si 101-200	Tumor Size (mm²) 101-200 201-300	> 301	Mean Tumor Size (mm²)
After 7 Weeks:•									
Control (saline)**	80	-	7	13%	0	2	-	m	245 ± 173
Irradiated CT26 only	2	6	7	30%	0	,7	4	944	180 ± 155
Irradiated CT26 mixed with unmodified fibroblasts	•	8	4	33%	0	8	-	-	170 ± 160
Irradiated CT26 mixed with LNCX-modified fibroblasts	2	e	7	30%	m	0	-	е	140 ± 142
Irradiated CT26 mixed with LNCX-IL2-modified fibroblasts	13	1	vo	54%		. n	-	-	ı 44
Irradiated CT26 mixed with LXSN-IL2-modified fibroblasts	12	4	00	33%	8	0	7	-	
Irradiated CT26 mixed with DCTK-IL2-modified fibroblasts	01	7	m	70%	-	7	0	0	41 ± 75

Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

One mouse in this arm developed an intraperitoneal tumor which was not measurable.

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These results demonstrate the feasibility of using genetically modified fibroblasts as a means of delivering cytokine gene therapy. In all experiments, the LNCX-L2 vector proved superior in preventing tumor establishment while the DCTK-IL2 vector was better in the induction of systemic protection against subsequent tumor challenges. These contrasting effects, although somewhat surprising, can be explained by the observation that the CMV promoter is turned off in vivo five days after implantation while the TK promoter remains active for a longer period of time. The implication of this finding is that to apply this method of gene therapy successfully we have to use promoters that result in high level, sustained expression of IL-2 in vivo in the transduced fibroblasts.

The data obtained from this research effort has important implications for all cytokines that have either direct or indirect anti-tumor effects. Furthermore, this data suggests that anti-tumor efficacy is IL-2 dose dependent. Hence, construction of vectors which result in higher levels of cytokine secretion will be a significant advance toward the application of this method of gene therapy.

Reference numbers in parenthesis in the above examples correspond to the following list of references and are incorporated herein by reference.

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References

- Gabrilove, J.L. et al., Monogr. J. Natl. Cancer Inst. <u>10</u>:73-7 (1990).
- 5 2. Kelso, A., Current Opinion in Immunology, 2:215-25 (1989).
 - 3. Borden, E.C. et al., Cancer, 65:800-14 (1990).
 - 4. Rosenberg, S.A. et al., Ann. Intern. Med., 108:853-864 (1988).
- 10 5. Lotze, M.T. et al., JAMA, 256:3117-3124 (1986).
 - 6. Pizza, G. et al., Lymphokine Research, 7:45-8 (1988).
 - 7. Sarna, G. et al., Journal of Biological Response Modifiers, 9:81-6 (1990).
- 15 8. Gandolfi, L. et al., Hepato-Gastroenterology, 36:352-6 (1989).
 - 9. Bubenik, J. et al., immunol. Letters, 19:279-82 (1988).
- 10. Bubenik et al., Immunol. Letters, <u>23:287-292</u>
 20 (1990).
 - 11. Fearon, E.R. et al., Cell, 60:387-403 (1990).
 - 12. Gansbacher, B. et al., J. Exp. Med., 172:1217-1224 (1990).
- 13. Watanabe, Y. et al., Proc. Natl. Acad. Sci., 86:9456-9460 (1989).

- 14. Tepper, R.I. et al., Cell, <u>57</u>:503-512 (1989).
- 15. Kriegler, M., Gene Transfer and Expression: A Laboratory Manual, Stockton Press (1990).
- 16. Rosenberg, S.A. et al., N. Eng. J. Med., 370 (1990).
 - 17. Cornetta, K. et al., Prog. Nucl. Acid Res. Mol. Biol., 36:311-22 (1989).
 - 18. Hoover, H.C. et al., Cancer Res., 44:1671-76 (1984).
- 10 19. Sobol et al. New Eng. J. Med. 316:1111-1117 (1987).
 - 20. Li Xu, et al., Virology, <u>171</u>:331-341 (1989)...

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Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention.

5 Accordingly, the invention is limited only by the following claims.

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WE CLAIM:

- A method of treating cancer in a patient comprising the stimulation of that patient's immune response against the cancer by immunizing said patient at a site other than an active tumor site with a formulation comprising tumor antigens and CE cells genetically modified to express at least one cytokine gene product.
 - The method of claim 1 wherein tumor cells previously isolated from said patient provide the tumor antigens.
 - 3. The method of claim 1 wherein the cytokine gene is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, and gamma-interferon.
 - 4. The method of claim 3 wherein one cytokine gene is interleukin-2.
 - 5. The method of claim 1 wherein at least one cytokine gene is transferred into cells to generate CE cells by recombinant methods.
 - 6. The method of claim 5 wherein the cytokine gene is present in an expression vector.
 - 7. The method of claim 6 wherein said expression vector additional contains a suicide gene.
- 8. The method of claim 5 wherein the CE cells are generated from fibroblasts and antigen-presenting cells.

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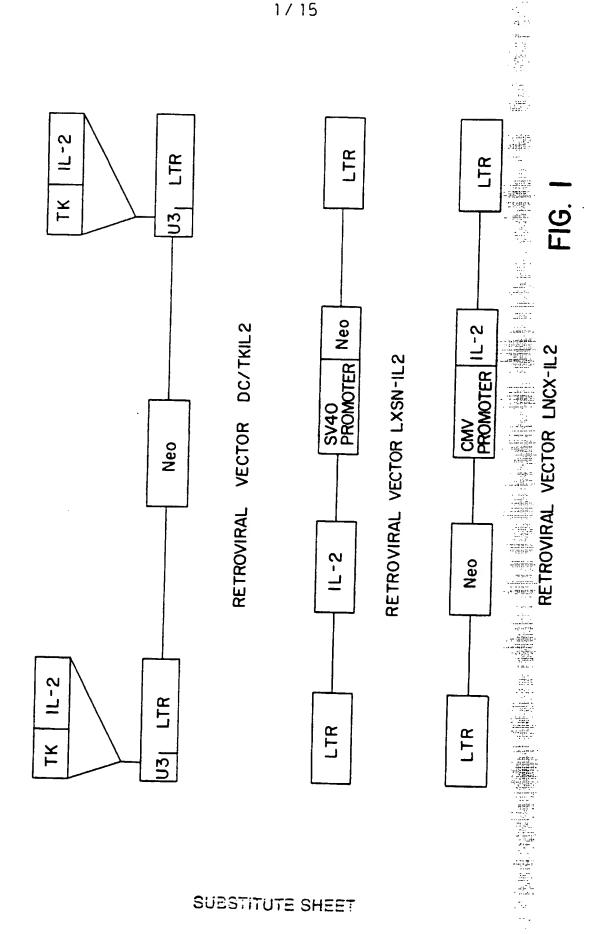
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- 9. A method for enhancing a patient's immune response to a cancer comprising:
 - a) isolating fibroblasts from said
 patient;
 - b) culturing said fibroblasts in vitro;
 - c) transducing said fibroblasts with a retroviral expression vector containing the gene coding for IL-2 and a gene coding for a tumor antigen in a retroviral expression vector, to express said tumor antigen and to express and secrete said IL-2 by said fibroblasts; and
 - d) immunizing said patient with said fibroblasts that express IL-2 at a level sufficient to enhance an immune response but low enough to avoid substantial systemic toxicity and that express said tumor antigen, at a site other than an active tumor site.
- 10. The method of claim 9 wherein said fibroblasts are further modified to express a suicide gene.
- 11. A composition for increasing a patient's immune response to tumor antigens comprising tumor antigens and CE cells genetically modified to express at least one cytokine gene product.
- 12. The composition of claim 11 wherein the cytokine gene is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, and gamma interferon.
- 13. The composition of claim 12 wherein one cytokine gene is interleukin-2.

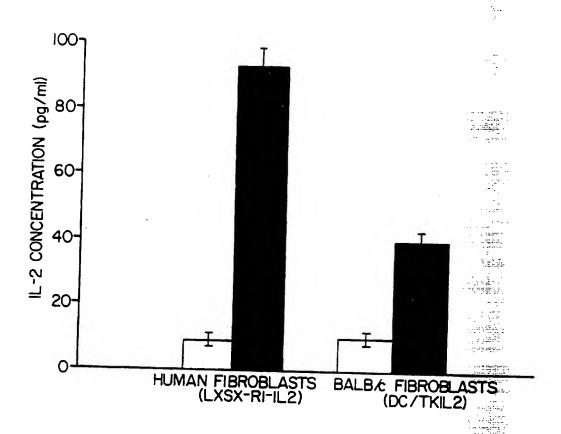
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14. The composition of claim 11 wherein each cytokine gene is expressed at a level sufficient to stimulate the immune response but low enough to avoid substantial systemic toxicities.

- 15. The method of claim 9 wherein in said transducing step said retroviral expression vector has a promotor causing sustained secretion of IL-2.
- 16. The method of claim 15 wherein said retroviral expression vector causes the secretion of at least four units of IL-2 per day for a period of ten days or longer.



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UNMODIFIED CELLS IL-2 TRANSDUCED CELLS

FIG. 2

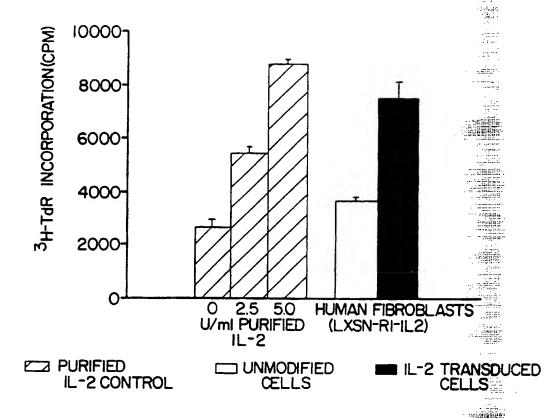
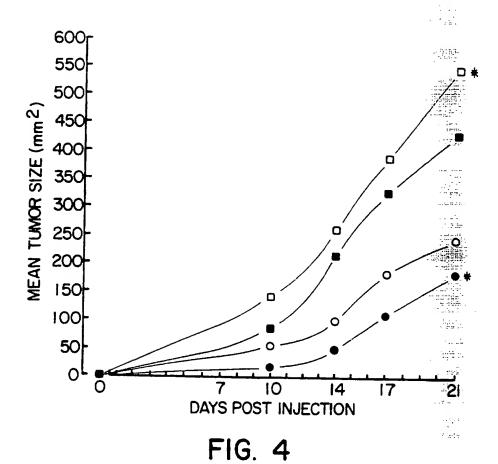


FIG. 3



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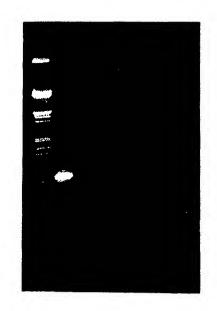
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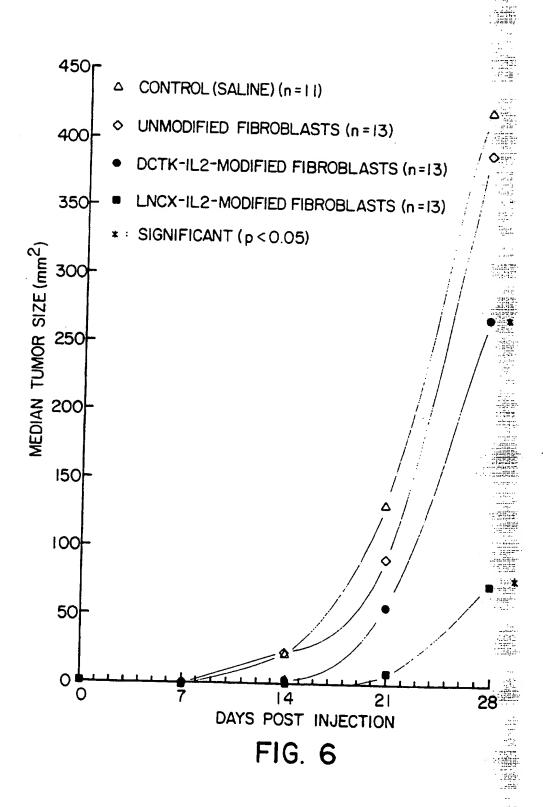
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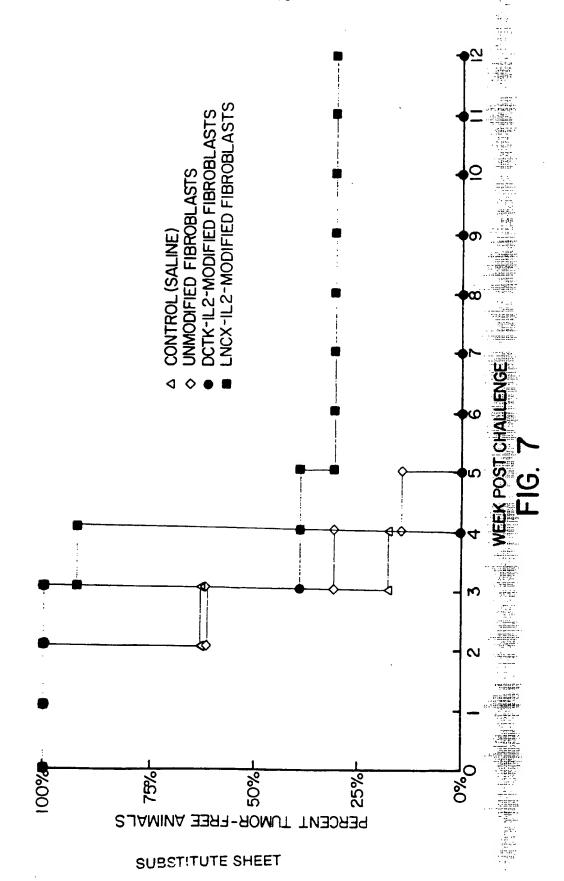
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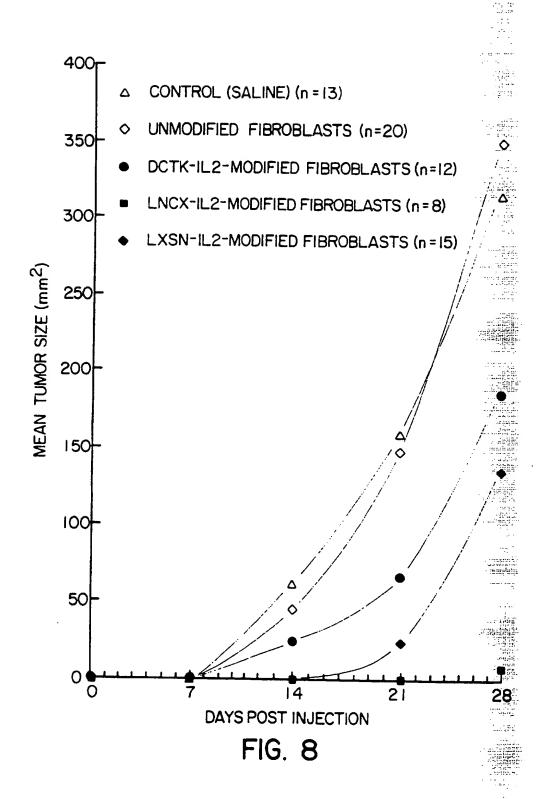
FIG. 5

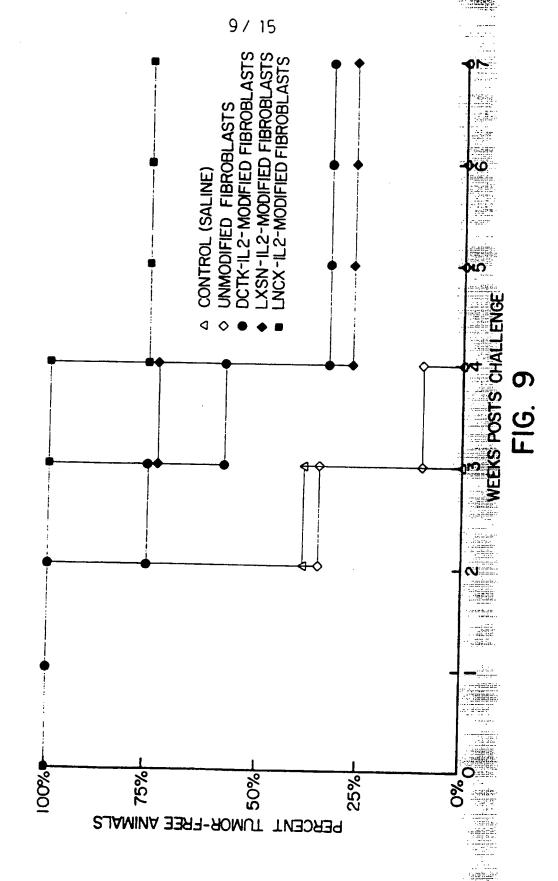


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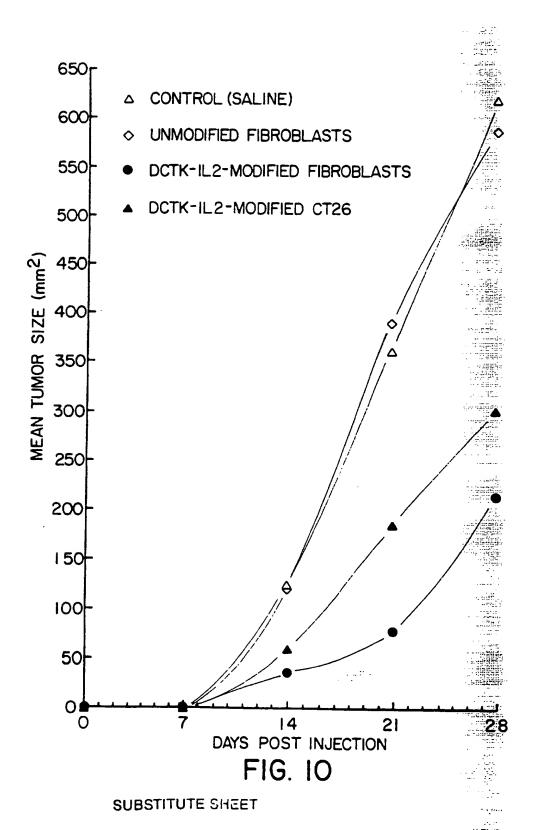
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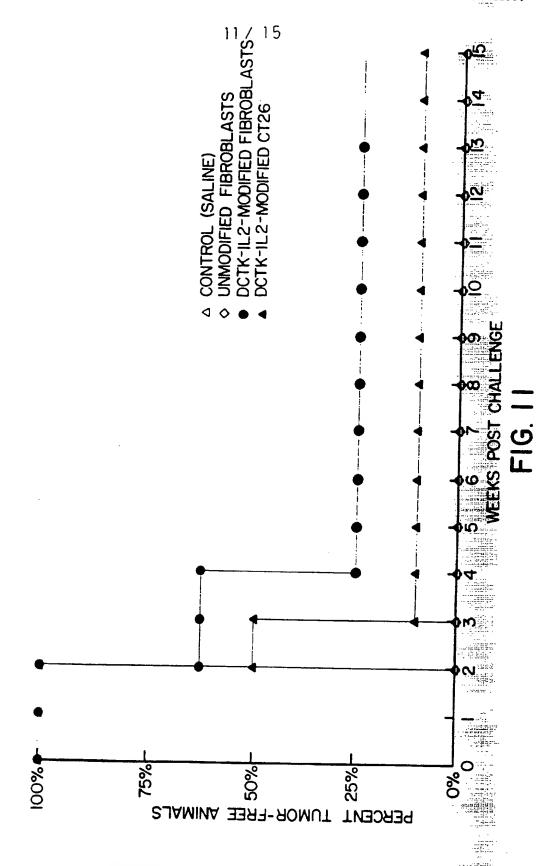




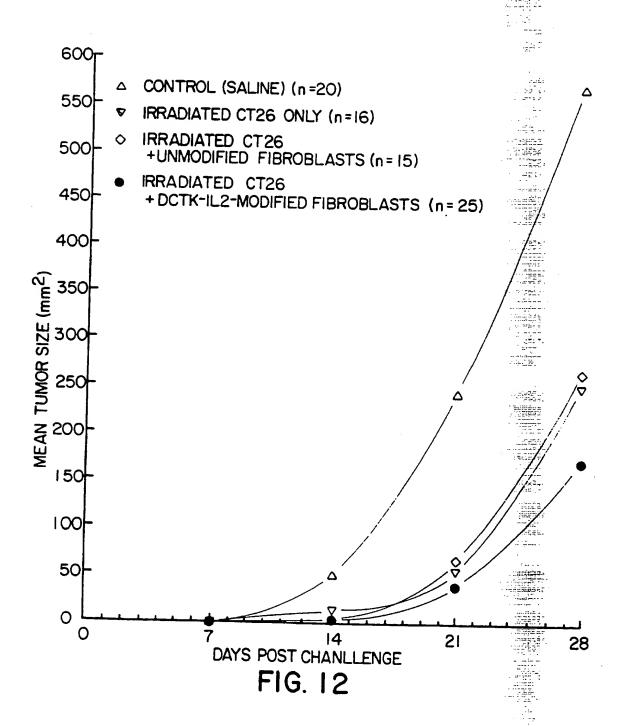


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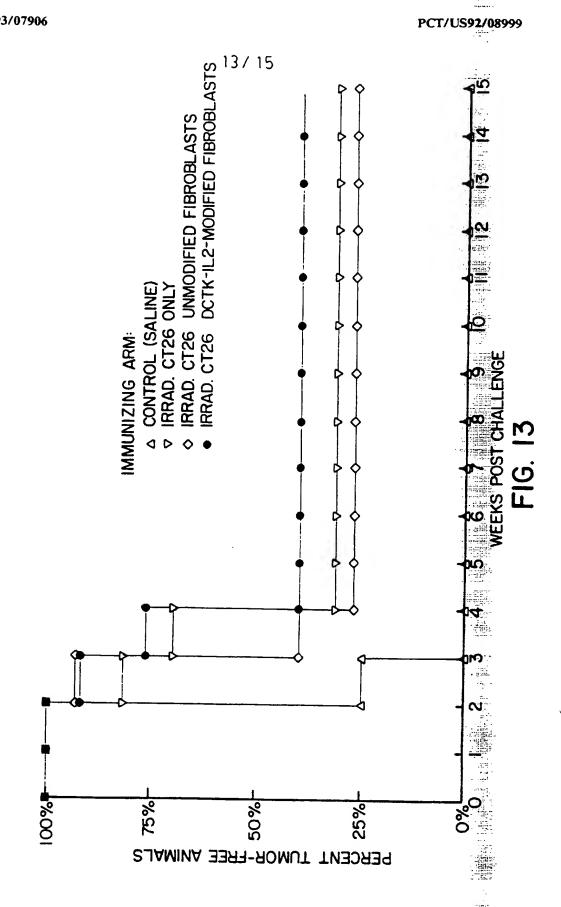




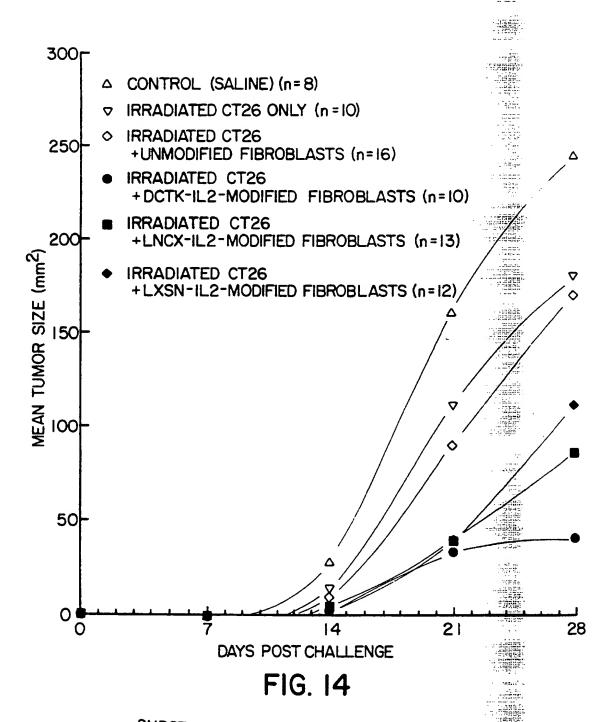
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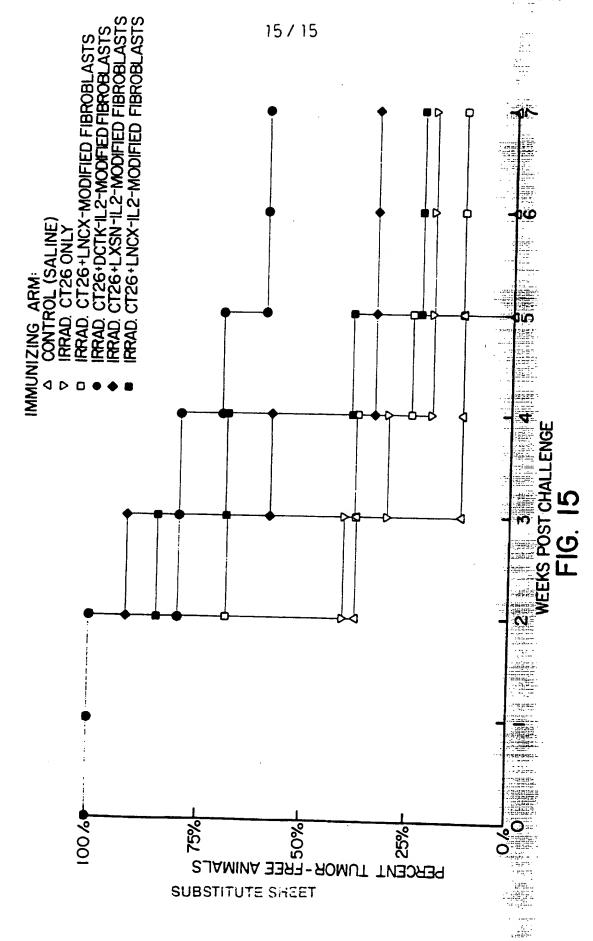
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INTERNATIONAL SEARCH REPORT

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<u>X</u>	Journal of Experimental Medicine, Volume 179		· ir more grad
Ÿ	Journal of Experimental Medicine, Volume 172,	issued October 1990, Gansbacher et al.	1-8. 11-14
	*Interleukin 2 Gene Transfer into Tumor Cells	Abrogates Tumorigenicity and Induces	9, 10, 15 , 16
	Protective Immunity", pages 1217-1224, see the	entire document.	
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Ÿ .	Cell, Volume 57, issued 05 May 1989, Tepper et :	II. Murine Interleukin-4 Displays Potent	1-3.5:6::8.11.12.14
•	Anti-Tumor Activity In Vivo", pages 503-512, se	the entire document.	4, 13
Y	Cell Valuma 60 insued 60 E t		
X	Cell, Volume 60, issued 09 February 1990, Fea	ron et al., "Interleukin-2 Production by	1.3-5.8-11-13
•	tumor cem sypasses I Helper Function in the	Generation of an Antitumor Response",	2, 6, 7, 14-16
	pages 397-403, see the entire document.		A THE PARTY OF THE
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1 1	Cancer Research, Volume 50, issued 15 Augus	t 1990, Ogura et al., "Implantation of	1-16
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INTERNATIONAL SEARCH REPORT

Inte. stional application No. PCT/US92/08999

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<u> </u>	Cancer Research, Volume 50, issued 15 December 1990, Gansbacher et al., "Retron	(Titalia)
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A. CLASSIFICATION OF SUBJECT MATTER: US CL :

424/93B, 93U, 89; 435/240.2, 320.1, 69.5, 69.51, 69.52; 935/65, 32, 12, 57, 70, 71